Association of YY1 With Maternal mRNAs in Oocyte mRNPs.

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Abstract

Early embryonic development in vertebrates is directed in part by maternal mRNAs expressed in oocytes and stored in cytoplasmic messenger ribonucleoprotein particles (mRNPs). Abundant evidence demonstrates the importance of mRNPs in embryonic development and in post-embryonic cellular function; however their characterization has been hampered by lack of suitable methodologies. The *Xenopus* oocyte has been the primary model system for studies of mRNPs. YY1 is a well-studied transcriptional regulatory factor that is sequestered in the oocyte cytoplasm and present entirely in cytoplasmic oocyte mRNPs. The objective of this thesis was to examine the biochemistry of YY1 association with maternal mRNA molecules in order to shed light on the role of YY1 in development and the poorly understood biology of oocyte mRNPs. The initial working hypotheses were that association of YY1 with mRNPs is dependent on sequence-specific RNA-binding activity and, therefore, that YY1 associates with a definite subset of maternal mRNA. A number of unique methods were developed in this study to address these hypotheses. RNA immunoprecipitation-DNA microarray (RIP-CHIP) analysis establishes that YY1 associates with a subset of mRNAs in the oocyte pool. A novel sequence-specific RNA-binding activity of the YY1 protein is demonstrated, and the RNA-binding activity of YY1 is shown to be required for its association with oocyte mRNPs *in vivo*. The functional roles of YY1 mRNA substrates are discussed in the context of embryological development and the biological function of YY1 in oocyte mRNPs. Extension of the experimental approaches developed in this thesis to the entire set of mRNP proteins would significantly advance our understanding of mRNP composition and heterogeneity, as well as the biological function of maternal mRNAs and mRNPs in development.

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A - adenosine;

ATP - adenosine triphosphate;

BSA - bovine serum albumin;

C - cytosine;

CBTF - CAT-box transcription factor;

CHO - Chinese hamster ovary;

CIRP2 - cold inducible RNA-binding protein 2;

CKII - casein kinase-II;

CMV - cytomegalovirus;

CRAS - coding region activating sequence;

dCTP - deoxycytidine triphosphate;

DEPC - diethylpyrocarbonate;

DMEM - Dulbecco's modified Eagle Medium;

dNTP - deoxyribonucleotide triphosphate;

DTT - dithiothreitol;

EDTA - ethylenediaminetetraacetic acid;

EMSA - electrophoretic mobility shift assay;

EST - expressed sequence tag;

FRGY2 - Frog Y-box Protein 2;

G - guanidine;

GAPDH - glyceraldehyde-3-phosphate dehydrogenase;

GO - gene ontology;

GRE - guanidine/uridine rich element;

HA - hemagglutinin;

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

IPTG - isopropyl β-D-1-thiogalactopyranoside;

IMAC - immobilized metal affinity chromatography;

IP - immunoprecipitation;

IVT - *in vitro* transcription;

MBT - mid-blastula transition;

MEM - minimal essential medium;

MES - 2-(N-morpholino)ethanesulfonic acid;

MLuV - Moloney murine leukemia virus;

mRNP - messenger ribonucleoprotein particle;

NTA - nitrilotriacetic acid;

NTP - nucleotide triphosphate;

OD - optical density;

oligo-dT - oligodeoxythymidine;

PAGE - polyacrylamide gel electrophoresis;

PBST - phosphate-buffered saline with tween-20;

PCNA - proliferating cell nuclear antigen;

PCR - polymerase chain reaction;

RAP55 - RNA-associated protein 55 kDa;

RIP-CHIP - RNA-immunoprecipitation/DNA microarray;

RNase - ribonuclease;

RPL3 - ribosomal protein L3;

SEC - size exclusion chromatography;

SDS - sodium dodecyl sulphate;

T - thymidine;

TCEP - tris(2-carboxyethyl)phosphine;

TEMED - N,N,N',N'-tetramethylethylenediamine;

U - uridine;

UTP - uridine triphosphate;

UTR - untranslated region;

UV - ultraviolet;

Vg1RBP - Vg1-RNA binding protein;

VLE - vegetal localization element;

Xstau1 - *Xenopus* staufen homologue-1;

YB-1 - Y-box protein 1;

YY1 - Yin-Yang 1;

1. Introduction - Embryonic development in metazoans is highly dependent on mRNA transcribed during oogenesis and translated in the embryo following fertilization to provide protein products required for differentiation and development (Brandhorst, 1985; Dworkin and Dworkin-Rastl, 1990; Evans and Hunter, 2005; Evsikov and Marin de Evsikova, 2009; Farley and Ryder, 2008; Gosden, 2002; Grainger, 1994; Heasman, 2006; Li et al., 2010; Pederson, 2006; Richter, 1991; Sardet et al., 2007; Semotok and Lipshitz, 2007; Tadros and Lipshitz, 2009). These transcripts are termed maternal mRNA in reference to their origin via transcription of the diploid genome of the female animal. These mRNA molecules persist through meiosis and fertilization and subsequently are utilized at specific developmental stages and cellular lineages (Bravo and Knowland, 1979; King et al., 2005; Laskey and Gurdon, 1974; Melton et al., 1989; Woodland et al., 1979). In all metazoans, stability and cell- and stage-specific translation of maternal mRNA throughout the protracted period of oogenesis and embryonic development is mediated by interaction with cytoplasmic RNA-binding proteins in complexes termed messenger ribonucleoprotein particles (mRNPs) (Smith and Richter, 1985; Sommerville, 1990; Spirin, 1966). The major model system for the study of maternal mRNA, mRNPs, and their role in embryonic development is the African Clawed Frog, *Xenopus laevis* (Dreyfuss et al., 2002; Grainger, 1994; Hake and Richter, 1997; Heasman, 2006; King et al., 2005; Kloc et al., 2001; Matsumoto and Wolffe, 1998; Melton et al., 1989; Richter, 1991; Richter et al., 1990; Sommerville, 1990; Sommerville, 1999). Yin-Yang 1 (YY1), a protein factor indispensable in *Xenopus* embryogenesis (Kwon and Chung, 2003; Morgan et al., 2004; Satijn et al., 2001), is sequestered entirely in cytoplasmic mRNP complexes in oocytes and early embryos *(Ficzycz et al., 2001; Ficzycz and Ovsenek, 2002)*. However, the mechanisms by which YY1 associates with maternal mRNA and mRNA content of YY1 containing mRNP complexes had not been

determined. The current study employs a number of novel approaches to demonstrate that YY1 associates with maternal mRNA *in vivo* via an RNA-binding activity of the YY1 protein and identifies the mRNA population present in YY1 containing mRNP complexes.

1.1 Embryonic Development of *Xenopus laevis* and Cytoplasmic Messenger Ribonucleoprotein Particles –

Xenopus laevis has long been a major model system in developmental biology. The large numbers of oocytes or embryos which can be obtained from a single female frog, the ease of *in vitro* fertilization, maintenance of oocytes and embryos in simple salt solutions, and development of the embryo outside the female have ensured the continued success of the *Xenopus* model system. Furthermore, the availability and size (0.05-2 mm) of *Xenopus* oocytes and embryos provide large quantities of nucleic acid and protein material for biochemical studies. Oogenesis and embryogenesis in *Xenopus* have been fully characterized morphologically (Dumont, 1972; Nieuwkoop and Faber, 1967). Dumont (Dumont, 1972) divided oogenesis into six distinct stages based on morphological criteria which are denoted by Roman numerals I to VI (embryonic stages are denoted by Arabic numerals). As oogenesis progresses from stage I to stage VI the oocyte increases from 50 μm to 1.3 mm in diameter, with an accompanying increase in cellular volume of 0.91 mm^3 (Dumont, 1972). This increased volume is occupied by a variety of materials which accumulate in the oocyte and are utilized later in development to provide amino acids, carbohydrates, and lipids; metabolic energy; cellular organelles and structural components; and, importantly, proteins required for embryogenesis and differentiation (Smith and Richter, 1985). Some protein products required later in embryogenesis accumulate in the oocyte, however, the majority of proteins utilized in early development arise by translation of mRNA transcribed in the oocyte and stored in mRNP complexes (Smith and Richter, 1985; Sommerville, 1990; Spirin, 1966).

In *Xenopus*, oogenesis typically lasts 6 – 8 months, during which time the oocyte remains arrested in a prolonged prophase of meiosis I whilst various materials required for later development accumulate. Maternal mRNA transcribed during this period is sufficient to direct development of the embryo up until the mid-blastula transition (MBT) at which point transcription from the zygotic genome begins (Davidson, 1986; Davidson and Hough, 1971). The role of maternal mRNA extends well beyond MBT with translation of maternal mRNA occurring in conjunction with gastrulation, neurulation, and organogenesis (Bravo and Knowland, 1979; Laskey and Gurdon, 1974; Woodland et al., 1979). This has been demonstrated most conclusively using androgenic haploid embryos produced by fertilization of enucleated *Xenopus laevis* oocytes with sperm of *Xenopus borealis,* wherein newly synthesized transcripts are derived from the *X. borealis* genome and can be distinguished from maternal *X. laevis* transcripts in northern blots (Woodland et al., 1979). In this system maternal mRNAs were found to be stable for long periods, then rapidly entered the unstable mRNA pool at MBT and gastrulation (Woodland et al., 1979). Bursts of protein synthesis were also observed in connection with MBT, gastrulation, and neurulation in embryos treated with pharmacologic inhibitors of transcription, indicating the required transcripts were recruited from the stored pool (Allende et al., 1974; Bravo and Knowland, 1979; Laskey and Gurdon, 1974). Transcription of maternal mRNA reaches its maximum level at stages III – IV of oogenesis, coinciding with the appearance of mature lamp brush chromosomes due to decondensation of nuclear chromatin (Ficq, 1970; Hill and Macgregor, 1980; Pardue and Gall, 1969; Thomas, 1970). Accumulation of RNA in the cytoplasm proceeds at a rate of 1.7-2.2 pg/min (Anderson et al., 1982) until the completion of oogenesis at stage VI when oocytes contain approximately 4.3 µg of RNA (Rosbash, 1974). Of the total amount of RNA in the mature oocyte, 0.7 – 1.0 % (~40 ng) is

poly-A+ RNA, while ribosomal RNA comprises the major portion of the remainder (Rosbash, 1974). Approximately 20 % of cytoplasmic mRNA in stage VI oocytes is present in polyribosomes, the other 80 % being present as stored mRNA in mRNPs (Smith and Richter, 1985). Messenger ribonucleoprotein particles contain polyadenylated nuclear transcripts and exclude transcripts from the mitochondrial genome, since studies show only synthetic mRNAs injected in the nucleus are capable of entering the mRNP pool (Braddock et al., 1994). Some mRNP proteins, for example Xp54 (see below) have been localized to active areas of transcription in immature oocytes by immunoelectron microscopy and immunocytochemical methods (Smillie and Sommerville, 2002; Weston and Sommerville, 2006). These data suggest assembly of mRNP complexes begins concomitant with transcription of maternal mRNA and complex formation is necessary for nuclear export of maternal mRNA destined for the mRNP pool (Braddock et al., 1994; Smillie and Sommerville, 2002; Weston and Sommerville, 2006).

A number of protein factors essential for *Xenopus* development have been identified in *Xenopus* oocyte mRNP particles, including four major proteins abundantly present in mRNP complexes: Xp54, FRGY2A, FRGY2B, and RAP55 (Belak et al., 2008; Belak and Ovsenek, 2007; Bouvet et al., 1995; Bouvet and Wolffe, 1994; Brzostowski et al., 2000; Evans and Hunter, 2005; Ficzycz et al., 2001; Ficzycz and Ovsenek, 2002; Ladomery et al., 1997; Lieb et al., 1998; Marello et al., 1992; Marnef et al., 2009; Matsumoto et al., 2000; Minshall and Standart, 2004; Minshall et al., 2001; Moore, 2005; Smillie and Sommerville, 2002; Sommerville, 1990; Tanaka et al., 2006; Weston and Sommerville, 2006; Yurkova and Murray, 1997), and a number of significantly less abundant proteins including CIRP2 (Matsumoto et al., 2000), CBTF (Brzostowski et al., 2000), p100 (Nakamura et al., 2010), Vg1RBP (Git and Standart, 2002; Kwon et al., 2002), Xstau1 (Allison et al., 2004), and YY1 (Ficzycz and Ovsenek, 2002). Some

of these, such as Vg1RBP and Xstau1, have been shown to be required for localization of substrate mRNAs (Allison et al., 2004; Git and Standart, 2002; Kwon et al., 2002), however, the precise developmental roles of the other known mRNP proteins remain poorly understood. The stoichiometry and physical structure of mRNPs are currently uncharacterized. Therefore, it is unknown if the high abundance of the major mRNP proteins is due to many molecules of these proteins associating with each mRNA while only one or a very few of the less abundant proteins associate with each transcript. Alternatively, the abundant mRNP proteins may be present ubiquitously in all mRNP particles while the other less abundant proteins are bound to only a subset of the total mRNAs in the mRNP pool, as is the case for Vg1RBP and Xstau1 (Allison et al., 2004; Git and Standart, 2002; Kwon et al., 2002). Importantly, the mechanisms by which specific mRNA molecules are recruited from the mRNP pool at specific developmental time points and in specific cell types is unknown. The currently accepted theory is that Xp54, FRGY2A/B, and RAP55 from the "core" compliment of mRNP proteins while other less abundant proteins likely bind a subset of mRNP-associated mRNA and underlie the spatiotemporal translational recruitment of specific mRNA molecules (Farley and Ryder, 2008; Marnef et al., 2009; Standart and Minshall, 2008; Tanaka et al., 2006; Weston and Sommerville, 2006).

Xp54 is the *Xenopus* homologue of the human DEAD-box ATP-dependent RNA helicase p54 (Ladomery et al., 1997). Mammalian p54 has roles in a number of processes in mRNA metabolism including transcription, mRNA splicing and editing, translation initiation, nuclear export, and degradation (Lorsch, 2002). In *Xenopus*, photocrosslinking of [α-^{32}P]ATP to mRNPs in solution resulted in covalent labelling of a 54 kDa polypeptide (Ladomery et al., 1997). Cyanogen bromide digestion of this polypeptide and partial sequencing of the resulting peptide

fragments permitted cloning of *Xenopus* p54 by PCR using degenerate primers (Ladomery et al., 1997). Subsequent sequence alignment analysis demonstrated that Xp54 belongs to the DEAD-box RNA helicase family (Ladomery et al., 1997) which includes human p54 (Lu and Yunis, 1992), mouse p54 (Akao et al., 1995), *Drosophila* ME31B (de Valoir et al., 1991), *S. pombe* Ste13 (Maekawa et al., 1994), and *S. cerevisiae* DHH1 (Strahl-Bolsinger and Tanner, 1993). Immunocytochemical studies indicate Xp54 is most abundant in the nucleus in early oocytes and becomes progressively localized to the cytoplasm as oogenesis proceeds (Smillie and Sommerville, 2002). Furthermore, Xp54 appears to associate with areas of active transcription on lamp brush chromosomes in early oocytes, suggesting it associated with maternal mRNA concomitant with its transcription and then accompanies these mRNAs during nuclear export and into mRNPs (Smillie and Sommerville, 2002). Density gradient centrifugation shows Xp54 co-sediments with FRGY2A/B (Ladomery et al., 1997) in an RNA-dependent manner and Xp54 has been detected in mRNPs isolated by oligo-dT cellulose chromatography of oocyte lysates (Tanaka et al., 2006).

FRGY2A and FRGY2B are germ-line specific members of the Cold-Shock Doman containing Y-box protein family which includes the well-characterized homologous human YB-1 and rabbit p50 proteins (Richter and Smith, 1984). The p50 protein was the first Y-box protein to be discovered and characterized, initially in rabbit reticulocytes where it regulates translation and stability of globin mRNA (Minich et al., 1993). FRGY2A and FRGY2B are closely related polypeptides (85 % homology) originally identified by SDS-PAGE analysis of isolated mRNP particles (Darnbrough and Ford, 1981), and may represent the products of pseudoalleles in *Xenopus (Murray et al., 1992)*. FRGY2A/B, their somatic paralogue FRGY1, and the human orthologue YB-1 possess dual functional roles, as transcription factors, and as cytoplasmic RNA-

binding proteins (Evdokimova et al., 2006; Evdokimova and Ovchinnikov, 1999; Evdokimova et al., 1995; Kohno et al., 2003; Minich and Ovchinnikov, 1992). Interestingly, this dual DNA- and RNA-binding activity is shared with the other mRNP proteins CBTF (Brzostowski et al., 2000) and YY1 (Belak et al., 2008; Belak and Ovsenek, 2007) (see below). In oocytes, FRGY2A/B bind to single-stranded RNA and have been shown to play an essential role in masking and translational silencing of maternal mRNA molecules (Bouvet and Wolffe, 1994; Deschamps et al., 1991; Deschamps et al., 1992; Marello et al., 1992; Murray, 1994; Murray et al., 1992; Ranjan et al., 1993; Tafuri and Wolffe, 1990; Tafuri and Wolffe, 1993a; Tafuri and Wolffe, 1993b; Yurkova and Murray, 1997). Maximum levels of FRGY2A/B are achieved by stage II of oogenesis, however the majority of the protein is present as the monomer (Tafuri and Wolffe, 1993b). Coinciding with the accumulation of maternal mRNA in stages III – IV, FRGY2A/B protein is progressively incorporated into mRNP particles, and by stage VI mRNPs contain the entire cellular compliment of FRGY2A/B (Tafuri and Wolffe, 1993b). Reconstitution experiments demonstrated FRGY2A/B represses translation of mRNA templates *in vitro (Kick et al., 1987; Matsumoto et al., 1996; Ranjan et al., 1993; Richter and Smith, 1984; Yurkova and Murray, 1997)*, and microinjection of oocytes with function-blocking anti-FRGY2A/B antibodies relieved translational silencing mediated by FRGY2A/B (Braddock et al., 1994; Gunkel et al., 1995). Collectively, these data demonstrate the role of FRGY2A/B in translational repression of stored maternal mRNA. FRGY2A/B and the current study focussing on YY1 represent similar paradigms since both were originally described as transcription factors that were subsequently discovered in *Xenopus* oocyte mRNPs.

RAP55 (RNA-associated protein 55 kDa) is the most recent major mRNP protein to be identified (Lieb et al., 1998). It appears to interact with maternal mRNA via two RGG domains

located in the C-terminal region of the protein (Lieb et al., 1998) although protein-protein interactions may also play a role in its association with maternal mRNA (Lieb et al., 1998; Tanaka et al., 2006). RAP55 is known to repress translation of substrate mRNA molecules in somatic cells by interacting with the cytoplasmic polyadenylation element binding protein (CPEB) and thereby blocking cytoplasmic polyadenylation and the resulting translational activation (Marnef et al., 2009; Yang et al., 2006). Tethering of MS2-RAP55 fusion proteins to mRNA synthesized *in vitro* resulted in reduced translation *in vivo* and *in vitro* suggesting a similar role for RAP55 in oocyte mRNPs *(Tanaka et al., 2006)*. Overall, evidence suggests that FRGY2A/B and RAP55 are primarily responsible for translational repression of mRNP-associated mRNA while Xp54 is inactive in mRNPs and plays a role in translational recruitment of associated mRNA molecules through activation of its helicase activity by phosphorylation (Ladomery et al., 1997; Minshall and Standart, 2004; Minshall et al., 2001; Sommerville and Ladomery, 1996).

The history of the discovery of Vg1RBP differs significantly from other mRNP proteins. Initially, comparison of cDNA libraries generated from mRNA isolated from animal and vegetal poles of the oocyte revealed the transcript Vg1 was localized to the vegetal pole (Rebagliati et al., 1985). Subsequent Northern blotting revealed Vg1 was highly concentrated in the vegetal pole of the oocyte and retained this localization following fertilization (Rebagliati et al., 1985). Vg1 was subsequently shown to be a strong inducer of endodermal tissues (Henry et al., 1996). Mutational analysis revealed deletion of a 340 nucleotide sequence in the 3'UTR of Vg1, later termed the vegetal localization element (VLE), resulted in disruption of endoderm formation due to mislocalization of Vg1 mRNA (Joseph and Melton, 1997; Mowry and Melton, 1992). This prompted the search for protein factors that might interact with the VLE sequence and potentially

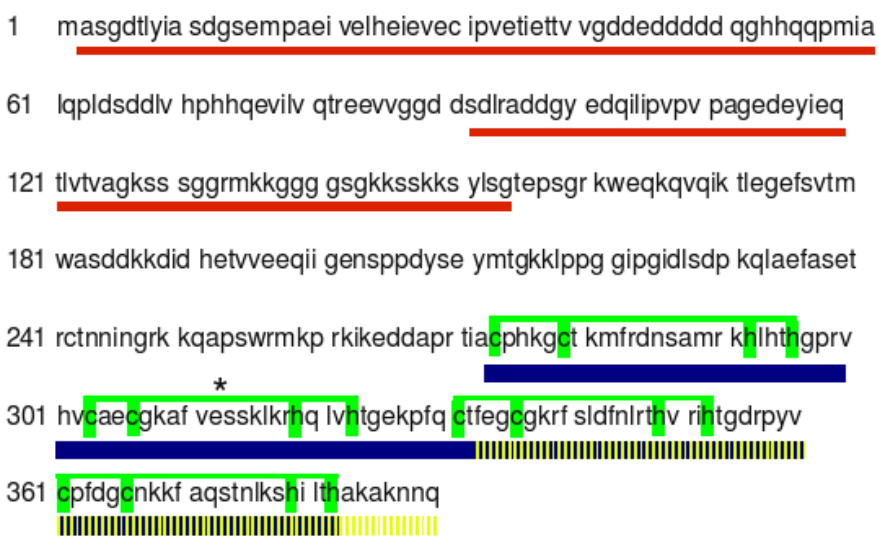
underlie localization of the Vg1 mRNA. Photocrosslinking followed by RNase T1 treatment of *in vitro* binding reactions containing oocyte lysate and radiolabelled VLE identified a 68 kDa polypeptide interacting with the VLE (Schwartz et al., 1992). This polypeptide was subsequently sequenced, characterized, and shown to underlie the localization of VLE-containing maternal mRNAs to the vegetal cytoplasm, and was ultimately given the designation Vg1-RNA binding protein (Vg1RBP) (Git and Standart, 2002). The identification of Vg1RBP in mRNPs was based on the search for protein factors responsible for a specific developmental effect, namely, the crucial localization of certain mRNAs to the vegetal cytoplasm of the oocyte. This is in contrast to the situation with other mRNP proteins, such as YY1. The mRNP proteins discussed above, and specifically YY1, are known to play essential roles in development (see below), however, the mechanism by which these effects are manifested, and the specific mRNA substrates potentially targeted and/or regulated during development through interaction with these factors is unknown. Therefore, the aim of the current study is to analyze the biochemical mechanism by which YY1 associates with maternal mRNA in mRNPs and identify the specific mRNA substrates of YY1 in mRNPs. This knowledge will provide a basis on which to further elucidate the role of YY1 in early vertebrate development.

1.2 YY1 Protein: Review of Literature to Date - Yin Yang 1 (YY1) is one of the most well-known transcription factors and the vertebrate protein has been the subject of over 750 scholarly articles. However, the role of YY1 in development has not been extensively studied. YY1 is a highly conserved transcription factor of the GLI-Kruppel family with functions in activation, repression, or initiation of transcription at a number of cellular and viral promoters depending on the cellular context (Galvin and Shi, 1997; Gordon et al., 2006; Ladomery and Dellaire, 2002; Shi et al., 1997; Thomas and Seto, 1999; Wang et al., 2006). The activity of

YY1 is modified by numerous interactions with other proteins including c-Myc (Shrivastava et al., 1993), Sp1 (Lee et al., 1994; Seto et al., 1993), the polycomb group protein EED (Satijn et al., 2001), cAMP response element binding protein (O'Connor et al., 1996), the viral protein E1A (Shi et al., 1991), retinoblastoma protein (Petkova et al., 2001), the GATA1 transcription factor (Rincon-Arano et al., 2005), and the specific activator YY1AP (Wang et al., 2004). YY1 is subject to posttranslational modifications such as glycosylation (Hiromura et al., 2003; Ozcan et al., 2010), acetylation (Yao et al., 2001), and phosphorylation (Becker et al., 1994). Regulation of the transcriptional activity of YY1 is also achieved through nucleocytoplasmic redistribution of the protein (Bernard and Voisin, 2008; Broyles et al., 1999; Favot et al., 2005; Krippner-Heidenreich et al., 2005; Oh and Broyles, 2005; Palko et al., 2004; Rylski et al., 2008; Seligson et al., 2005; Shestakova et al., 2004; Slezak et al., 2004). The main structural features of YY1 are shown diagrammatically in Figure 1. YY1 contains four C2H2-type zinc fingers near the C-terminus responsible for YY1 DNA binding activity (Austen et al., 1997), a bipartite activation domain near the N-terminus (Austen et al., 1997), and a transcriptional repression domain near the C-terminus (Bushmeyer et al., 1995). The *Xenopus laevis* homologue is a 43 kDa, 373 amino acid protein sharing

Figure 1 - Domain Structure of YY1. The amino acid sequence of the *Xenopus* YY1 protein is presented in FASTA format and important structural features are highlighted. The Zinc finger/DNA binding region is underlined in blue. The four C2H2 type zinc fingers are individually highlighted in green along with the key cysteine and histidine residues responsible for coordination of zinc ions. The second zinc finger shows close homology with the known RNA-binding protein TFIIIA and is indicated with an asterisk. The N-terminal bipartite transcriptional activation domain is underlined in red. The C-terminal transcriptional repression domain overlaps the last two zinc fingers and is underlined in dashed yellow.

Figure 1


1 masgdtlyia sdgsempaei velheievec ipvetiettv vgddeddddd qghhqqpmia
61 lqpldsddlv hphhqevilv qtreevvggd dsdlraddgy edqilipvpv pagedeyieq
121 tlvtvagkss sggrmkkggg gsgkksskks ylsgtepsgr kweqkqvqik tlegefsvtm
181 wasddkkdid hetvveeqii gensppdyse ymtgkklppg gipgidlsdp kqlaefaset
241 rctnningrk kqapswrmkp rkikeddapr tiacphkgct kmfrdnsamr khlhthgprv
301 hvcaecgkaf vesskikrhq lvhtgekpfq ctfegcgkrf sldfnlrthv rihtgdrpyv
361 cpfdgcnkkf aqstnlkshi lthakaknnq

a high degree of sequence and structural conservation with mammalian YY1 (86 % similar relative to Human YY1) (Pisaneschi et al., 1994).

Cytoplasmic redistribution of YY1 was first observed in connection with vaccinia virus infection (Broyles et al., 1999). Immunofluorescence, immunoprecipitation, and gel-shift analyses indicate YY1 is rapidly redistributed to the cytoplasm following vaccinia virus infection where it participates in cytoplasmic transcription of viral genes in concert with viral protein factors (Broyles et al., 1999; Oh and Broyles, 2005; Slezak et al., 2004). Immunocytochemical analyses of CHO and HeLa cells demonstrate YY1 is predominantly a cytoplasmic protein during G1- and G2-phases of the cell cycle and undergoes a DNA-replication dependent shift in localization to the nucleus during S-phase (Palko et al., 2004). Accumulation of YY1 in the nucleus at the onset of S-phase appears to correspond to transcriptional regulation of replication-dependent histone and other cell-cycle dependent genes by YY1 (Palko et al., 2004). Krippner-Heidenreich *et al.* also found YY1 to be predominantly cytoplasmic in HeLa cells, and found YY1 is rapidly translocated to the nucleus following application of apoptotic stimuli (Krippner-Heidenreich et al., 2005). YY1 has also been observed to be primarily cytoplasmic in quiescent (G0) 3T3 fibroblasts (Shestakova et al., 2004). A study of expression and localization of YY1 in 1364 prostate cancer tumour samples revealed statistically significant higher overall levels of YY1 expression and higher levels of cytoplasmic YY1 than in control tissues (Seligson et al., 2005). Decreased levels of nuclear YY1 and increased cytoplasmic abundance of the protein also correlated with decreased patient survival rates and higher (more severe) tumour morphological grading (Seligson et al., 2005). Recently, Favot *et al.* studied YY1 cellular distribution in connexion with neonatal pulmonary hypertension in a newborn piglet model system (Favot et al., 2005). It was found that YY1 was redistributed to the cytoplasm of vascular

smooth muscle cells following hypoxia with a corresponding increase in expression of smooth muscle-specific markers and inhibition of cell proliferation (Favot et al., 2005). Studies on chicken retinal cells show YY1 is highly expressed in photoreceptor cells and undergoes shifts in localization from nucleus to cytoplasm in response to light (Bernard and Voisin, 2008). Extensive studies of YY1 expression and subcellular localization have been undertaken in the brain of adult rats (Rylski et al., 2008). Immunohistochemical analyses reveal YY1 is nuclear in most types of neural cells, but is present in the cytoplasm of stellate and basket cells of the molecular layer of the cerebellum which exhibit considerable plasticity (Rylski et al., 2008). Taken together, the literature suggests that redistribution of YY1 to the cytoplasm is a common mechanism used by cells to regulate its transcriptional activity. None of these studies indicate a role for cytoplasmic YY1 beyond sequestering it from genomic DNA as a mechanism for controlling its transcriptional activity. Given the drive toward efficiency implicit in evolution, it is not uncommon to discover multiple, sometimes unrelated, functions of a single protein. Studies directed at elucidating the role of YY1 in early development have suggested a non-transcriptional role for cytoplasmic YY1 in development.

The first studies of YY1 in development were carried out by Donohoe *et al.* who created YY1 knockout mice and established the essential role for YY1 in vertebrate development (Donohoe et al., 1999). Homozygous YY1 knockout embryos implanted successfully in the uterine wall but failed to undergo gastrulation, indicating YY1 knockout results in embryonic death around the time of implantation (Donohoe et al., 1999). Heterozygous embryos survived implantation but went on to display a number of defects such as lowered implantation efficiency, neurulation defects, and exencephaly (Donohoe et al., 1999). Analysis of the nucleocytoplasmic distribution of YY1 in mouse development revealed it is entirely cytoplasmic in oocytes and in

zygotes until the 2-cell stage when transcription from the zygotic genome is activated (Donohoe et al., 1999). YY1 was present in both nuclear and cytoplasmic compartments of cells of the blastocyst, inner cell mass, and trophoectoderm in stage E3.5 embryos (Donohoe et al., 1999). Whilst this study conclusively demonstrates a requirement for YY1 in mouse development, the researchers did not determine its transcriptional activity through development.

Initial studies on YY1 in early development of *Xenopus* were performed by Ficzycz *et al.* with the aim of elucidating factors involved in histone gene expression in early development (Ficzycz et al., 1997). Photocrosslinking experiments using late stage *Xenopus* embryo extracts revealed the presence of an 85 kDa polypeptide bound the H3.2 gene CRASα promoter region (Ficzycz et al., 1997). This polypeptide was later identified as *Xenopus* YY1 (Ficzycz et al., 2001). Analysis of DNA-binding activity through development revealed that while YY1 protein levels remained relatively constant throughout development, YY1 DNA-binding activity was only present in early oocytes and post-MBT embryos (Ficzycz et al., 2001). Subsequently, YY1 transcriptional activity was investigated through development using reporter plasmid constructs microinjected in one-cell embryos (Ficzycz et al., 2001). It was found YY1 did not significantly stimulate or repress transcription even in post-MBT embryos where YY1 DNA-binding activity had been demonstrated previously (Ficzycz et al., 2001). This finding prompted analysis of the nucleocytoplasmic distribution of YY1 in oocytes and embryos. YY1 was found to be entirely cytoplasmic from oocyte stage III, through fertilization and MBT, and into embryonic development until at least neurulation (stage 13) (Ficzycz et al., 2001). Subcellular fractionation confirmed that YY1 was present only in the cytoplasm of neurula stage embryos (Ficzycz et al., 2001).

Knowledge of previously identified factors such as FRGY2A/B (Bouvet and Wolffe,

1994; Deschamps et al., 1992; Marello et al., 1992; Matsumoto et al., 1996; Matsumoto and Wolffe, 1998; Murray, 1994; Sommerville and Ladomery, 1996; Tafuri and Wolffe, 1993a; Yurkova and Murray, 1997) and CBTF (Brzostowski et al., 2000) known to play dual roles as transcription factors and as cytoplasmic RNA-binding proteins led to the theory that YY1 may be present in mRNP particles in the cytoplasm (Ficzycz et al., 2001). The second zinc finger knuckle of YY1 is highly homologous with the RNA-binding zinc finger knuckles of the double stranded RNA binding protein TFIIIA, particularly in the spacing of key cysteine and histidine residues (Ficzycz and Ovsenek, 2002). This homology suggested the possibility that YY1 possesses an as-yet uncharacterized RNA-binding activity which could account for its association with maternal mRNA (Ficzycz and Ovsenek, 2002). Furthermore, it was observed that treatment of cytoplasmic lysates from stage VI oocytes with RNase A resulted in acquisition of specific YY1 DNA-binding activity (Ficzycz and Ovsenek, 2002). The presence of YY1 in mRNP particles was confirmed by oligo-dT cellulose chromatography of oocyte lysates, with retention of YY1 on oligo-dT cellulose matrix being abolished by treatment of lysates with RNase A (Ficzycz and Ovsenek, 2002). Analysis of lysates by size exclusion chromatography demonstrated YY1 was present in complexes with an average apparent molecular mass of 480 kDa and co-eluted with the known mRNP protein FRGY2A (Ficzycz and Ovsenek, 2002). Size exclusion analysis also revealed that RNase treatment disrupted mRNPs and released YY1 monomers (Ficzycz and Ovsenek, 2002). Microinjection of RNase A directly into the cytoplasm was effective in disrupting mRNPs and unmasking YY1 DNA-binding activity but did not result in nuclear translocation of YY1, indicating nuclear exclusion of YY1 is not dependent on interaction with maternal mRNA (Ficzycz and Ovsenek, 2002). Oligo-dT cellulose chromatography experiments using embryo lysates reveal that YY1 becomes associated with

mRNPs during oocyte stages III – IV, concomitant with accumulation of maternal mRNA, and persist in the embryo until neurulation, at which time YY1 appears as a monomer but remains restricted to the cytoplasm (Ficzycz et al., 2001; Ficzycz and Ovsenek, 2002; Ficzycz, 2003). It is therefore likely that the nucleocytoplasmic redistribution of YY1 as a mechanism for regulating its transcriptional activity is distinct from its potential role in mRNPs.

Several studies have subsequently looked at YY1 in *Xenopus* development. Satijin *et al.* overexpressed YY1 in embryos and observed induction of an ectopic neural axis (Satijn et al., 2001). This work was published contemporaneously with the work of Ficzycz *et al.* and so there was no opportunity for Satijin *et al.* to consider potential YY1-RNA interactions in interpreting their results. Ficzycz *et al.* have shown that YY1 is restricted to the cytoplasm at the time of neural axis formation using biochemical, immunohistochemical, and reporter-construct based approaches (Ficzycz et al., 2001; Ficzycz and Ovsenek, 2002; Ficzycz, 2003). This is difficult to reconcile with the reports of transcriptional activity for YY1 made by Satijin *et al.* Given that Satijin *et al.* overexpressed both YY1 and its putative cofactor EED, did not analyze their subcellular location, and did not definitively demonstrate transcriptional activity of YY1 via reporter constructs, the precise relevance of this work to the *in vivo* situation is currently unclear (Satijn et al., 2001). Kwon *et al.* used morpholino-modified antisense oligonucleotides to probe the function of YY1 in development (Kwon and Chung, 2003). Microinjection of YY1-directed morpholinos (YY1-MO) resulted in anterior/posterior patterning defects and alterations in levels neural tissue specific markers (Kwon and Chung, 2003). The results of morpholino experiments in *Xenopus* are notoriously difficult to interpret, with non-specific effects most often manifesting as defects in neurulation (Eisen and Smith, 2008; Heasman, 2002). In the study by Kwon *et al.* the authors fail to convincingly show that microinjection of YY1-MO actually resulted in a

decrease in YY1 protein or an alteration in YY1 DNA-binding or transcriptional activity (Kwon and Chung, 2003). While the authors localized high levels of protein to the neural crest and neural tube of the developing embryo, they did not determine its subcellular location (Kwon and Chung, 2003). Again, the effects described by Kwon *et al.* seem to occur at a time when Ficzycz *et al.* describe an absence of YY1 transcriptional activity. The work of Latinkic *et al.* constitutes the only other study of YY1 in *Xenopus* development (Latinkic et al., 2004). Latinkic *et al.* convincingly demonstrated the involvement of YY1 transcriptional activity in regulation of the cardiac-specific MLC2 gene (Latinkic et al., 2004). However, this activity takes place in embryonic stages well past neurulation, and beyond those analyzed by Ficzycz *et al.* at a developmental stage when YY1 is likely present in the nucleus (Latinkic et al., 2004). At best, these studies taken together suggest a role for mRNP-associated YY1 in early *Xenopus* development; at worst, they certainly do not preclude such a role. In either case, they certainly point to the need for further elucidation of the precise role of YY1 in embryogenesis prior to neurulation.

1.3 RIP-CHIP Analysis: Background and Overview – RNA immunoprecipitation/DNA microarray analysis (RIP-CHIP) is a methodology developed relatively recently for identifying the RNA present in RNA-protein complexes in a variety of experimental contexts (Baroni et al., 2008; Jain et al., 2010; Keene et al., 2006). Essentially, the approach involves immunoprecipitation of a target RNA-binding protein, followed by isolation and labelling of the associated RNA. This immunoprecipitated RNA can then be analyzed, and the RNAs present identified, by hybridization to an appropriate oligonucleotide DNA-microarray. RIP-CHIP has previously been used to identify microRNA targets in cancer cells, mammalian brain development, and during viral infection (Dolken et al., 2010; Nelson et al.,

2010; Tan et al., 2009; Wang et al., 2010a; Wang et al., 2010b). RIP-CHIP has been used to analyze the population of transcripts bound by the histone stem-loop binding protein (Townley-Tilson et al., 2006), and to identify the RNA substrates of the chloroplast protein Whirly1 in barley (Melonek et al., 2010). Recently, RIP-CHIP has been applied in developmental biology to determine the turnover of mRNAs in muscle-cell precursors (Lee et al., 2010). Lee *et al.* have used RIP-CHIP to identify dynamic association of mRNAs with the CUGBP1 protein in myoblasts (Lee et al., 2010). Important findings included the identification of a subset of transcripts containing GU-rich sequences (termed GRE's) rapidly degraded in myoblasts expressing CUGBP1 (Lee et al., 2010). Included in this group of GRE containing transcripts were a number of transcripts encoding cell-cycle control proteins and intracellular transport, as well as MyoD1 and MyoG, encoding factors important in terminal differentiation of muscle tissue (Lee et al., 2010). These observations led the authors to conclude that CUGBP1 mediates rapid decay of both transcripts required only transiently in the cell (i.e. those for cell-cycle control proteins) as well as transcripts for factors such as MyoD1 and MyoG that stimulate differentiation (Lee et al., 2010). CUGBP1 was previously known to decline during myogenic differentiation. The current data allowed the authors to hypothesize that decline of CUGBP1 results in stabilization of MyoD1 and MyoG transcripts, whose protein products in turn drive myogenesis (Lee et al., 2010). This hypothesis was then verified using antisense knockdown of CUGBP1, which resulted in accumulation of MyoD1 and MyoG transcripts (Lee et al., 2010). The foregoing example illustrates the utility of RIP-CHIP analysis in elucidation of the biological roles of RNA-binding proteins. Lee *et al.* were able to use information obtained from RIP-CHIP analysis of substrates of CUGBP1, a protein known previously to decline specifically during differentiation of myoblasts, to directly determine the likely role of CUGBP1 in

myogenesis. They were then able to directly test this hypothesis, resulting in significant insight into an important developmental process.

RIP-CHIP analysis is an important, novel methodology for determining the RNA substrates of RNA binding proteins, particularly when there can be many thousands of substrate RNAs present, such as in mRNPs. Here, RIP-CHIP is used to definitively identify *in vivo* substrates of the YY1 protein, providing insight into the putative biological role of YY1 in mRNPs. RIP-CHIP relies on two pre-existing factors: First, a DNA microarray for the organism in question must be available. This has been achieved for *Xenopus*, particularly with the production of the *Xenopus* Genome 2.0 microarray by Affymetrix®. Despite the term "Genome" in the title, the *Xenopus* Genome 2.0 array is not, in fact, a whole-genome array, as it does not include intergenic regions or introns. This array includes 32,400 individual oligonucleotides ("spots") representing approximately 29,900 *Xenopus laevis* transcripts and ESTs. The second major requirement for useful RIP-CHIP experiments is adequate sequencing coverage for the model organism being used. With *Xenopus laevis* this is somewhat lacking. Sequencing of the *X. laevis* genome is still underway, and is complicated by the pseudotetraploidy of *X. laevis*, making alignment of sequenced genomic fragments extremely difficult (Bowes et al., 2010). Of all transcripts and ESTs represented on the array, only about 20 % are annotated, constituting transcripts for named, characterized proteins. A large part of the problem arises from the fact the *X. laevis* was one of the latest organisms to be used in high-throughput analyses such as genomics, transciptomics, and proteomics (Bowes et al., 2010). It will undoubtedly take some time for all the *Xenopus* homologues of known mouse, human, and rat genes to be identified. Additionally, *X. laevis* sequences have not been annotated with Gene Ontology (GO) terms, and so high-throughput methods for determining common functional features for large sets of

mRNAs is also problematic (Bowes et al., 2010). One way around this difficulty which is used in the current study was to retrieve the GO terms for the mouse homologue of each of the *Xenopus* transcripts; however this can only be applied to *Xenopus* transcripts whose protein products have been named and characterized. Despite these difficulties, RIP-CHIP was able to identify 417 named and characterized transcripts associated with YY1, demonstrating the utility of the approach. Furthermore, the dataset obtained can be used far into the future, to extract even more information as *Xenopus laevis* genomics and transcriptomics improve.

1.4 Relevance and Objectives –The importance of mRNP complexes in development cannot be overstated given that all developmental processes occurring in the *Xenopus* embryo are governed by maternal mRNA stored mRNPs until activation of the zygotic genome at MBT (Davidson, 1986) and beyond (Bravo and Knowland, 1979). Moreover, maternal mRNA continues to play a crucial role in later developmental events such as gastrulation, neurulation, and terminal differentiation of various cells and tissues (Bravo and Knowland, 1979; Davidson, 1986). Although the importance of mRNPs in vertebrate development has been known for almost 40 years (Ficq, 1970; Smith and Richter, 1985; Spirin, 1966), little is known about their structure or function. The mechanisms by which specific mRNP proteins associate with maternal mRNA and the population of maternal mRNAs which associate with mRNPs and individual mRNP proteins is unknown. One aim of the current study is to advance knowledge about the biochemistry and mRNA content of mRNPs, focussing on YY1 specifically. The YY1 protein is a well-studied transcription factor which has subsequently been shown to be a component of oocyte mRNPs. Rather than exhibiting a role in transcriptional regulation of early development, it would appear that YY1 has some biochemical activity in the cytoplasm. This is evidenced by the studies described above which appear to show effects of YY1 on development during periods

when it has been shown to be sequestered in cytoplasmic mRNPs. It is hypothesized based on structural analysis (Ficzycz and Ovsenek, 2002) that the YY1 protein possesses RNA-binding activity. It is further postulated that RNA-binding activity underlies association of YY1 with maternal mRNA and with mRNPs *in vivo.* Finally, a RIP-CHIP approach is employed to identify potential mRNA substrates of YY1 in mRNPs. It is hypothesized YY1 will not associate with transcripts from the mitochondrial genome or with mRNA undergoing active translation in the oocyte, consistent with previous data regarding the mRNA content of mRNPs (Braddock et al., 1994; Smith and Richter, 1985; Woodland et al., 1979).

The current study addresses these hypotheses as follows: The YY1 protein was bacterially expressed, and conditions for its efficient purification and renaturation were established. *In vitro* analyses using recombinant YY1 revealed a high-affinity RNA binding activity, with a preference toward U-rich substrate RNA. Subsequent experiments using endogenous YY1 isolated from oocytes confirm *in vitro* findings obtained with recombinant protein. Microinjection of U-rich competitor RNA into oocytes selectively blocked association of YY1 with mRNPs, indicating RNA-binding activity is required for association with maternal mRNA in mRNPs *in vivo.* In a novel application of the RIP-CHIP methodology, a specific antibody was used to immunoprecipitate YY1-mRNA complexes and isolated mRNAs were identified by DNA-microarray analysis. This constitutes the most comprehensive analysis of the activity of any mRNP protein undertaken thus far, conclusively demonstrating that YY1 associates with a subset of maternally transcribed mRNAs by direct RNA-binding activity. Furthermore, this is the first instance in which the actual, *in vivo* mRNA substrates of any of the known mRNP proteins have been identified. Determination of the mRNA population present in YY1-mRNPs is a tremendous leap forward in understanding the role of YY1, maternal mRNA,

and mRNP complexes in control of early embryogenesis.

2. Materials and Methods

2.1 General Procedures

2.1.1 Oocyte Manipulations, Microinjection, and Preparation of Lysates – All experiments involving animals were carried out according to *The Guide for the Care and Use of Laboratory Animals* and were approved by the Ethics Committee of the University of Saskatchewan. Oocytes were isolated from the ovaries of adult female *Xenopus laevis* (Boreal) and maintained in OR2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 5 mM HEPES (pH 7.6) at 18°C. Stage VI oocytes were selected according to Dumont (Dumont, 1972). Microinjections were performed using a Narashige® model IM-300 microinjector. For nuclear microinjection of DNA, 20 ng plasmid was injected in 20 nL of 0.1 M KCl at a pressure of 12 psi. For cytoplasmic microinjections either 20 pg of RNase A or 5 pmol of the indicated RNA oligonucleotide were injected in 20 nL of 0.1 M KCl at 12 psi of pressure. Each injection needle was individually calibrated and at the indicated pressure injection rates were 0.1 nL/ms ± 20%. Lysates were prepared by homogenizing 100 oocytes in 1 mL of mRNP buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM DTT, 1X Protease Inhibitor Cocktail (Sigma), 100 U/mL RNase inhibitor (Fermentas)), except that RNase inhibitor was omitted for experiments where lysates were treated with RNase A. Lysates were centrifuged at 15,000 X g for 12 min at 4°C. Supernatants were removed and centrifuged a second time and supernatants collected using care not to disturb the upper yolky layer. Total oocyte RNA was isolated by homogenization of 100 oocytes in 1 mL Trizol® (Invitrogen) and processed according to the manufacturer's directions.

2.1.2 Polymerase Chain Reaction – All PCR reactions were conducted under identical conditions. Reactions contained 5.0 µL of 10X Taq Buffer (Fermentas), 2.5 mM

$MgCl_2$, 250 nM forward and reverse primers, 250 µM dNTPs, 1.0 µL template DNA, and 5 U Taq DNA polymerase (Fermentas) in a final volume of 50 µL. Reactions were cycled using an MJ Minicycler® using the following program: 95 °C/5 min; followed by 30 cycles of: 95 °C/45 sec, 58 °C/45 sec, and 72 °C/2 min. For PCR reactions with products less than 500 bp in length, the time of extensions were shortened to 1 min at 72 °C. Fidelity and specificity of all primers was verified by electrophoresis of PCR reactions on TAE-agarose gels.

2.1.3 Western Blotting Procedures, Antibodies, and Silver Staining of SDS-PAGE Gels – SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 1 mm gels containing 10% (w/v) of 29:1 acrylamide:bisacrylamide according to standard protocols (Laemmli, 1970). For Western transfer PVDF membranes (Bio-Rad) were pre-wet in methanol and equilibrated with transfer buffer (192 mM glycine, 25 mM Tris base, 10% (v/v) methanol). Transfers were carried out using a Bio-Rad semi-dry transfer apparatus at 25 V for 30 min at room temperature. Membranes were blocked overnight in 5% milk/PBST (5 % (w/v) skim milk powder, 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.76 mM KH_2PO_4, 0.1% (v/v) Tween-20, pH 7.4). Primary antibodies were applied at the concentrations indicated for 2 h at room temperature in 5% milk/PBST. Membranes were washed 3 X 30 min in 5% milk/PBST, then immersed in secondary antibody at the indicated concentration in 5% milk/PBST for 1 h at room temperature. Finally, membranes were washed 2 X 10 min in 5% milk/PBST then 2 X 5 min in PBST. Blots were treated with chemiluminescence reagent (PerkinElmer Life Sciences) for 1 minute and exposed to BioMax MS Lite film (Kodak). Antibodies used were: Rabbit anti-YY1 (Santa Cruz Biotechnology, Cat. #SC-281) at 1:1000; mouse anti-PCNA (Santa Cruz Biotechnology, Cat. #SC-56) at 1:5000; rabbit anti-His6 (Santa Cruz Biotechnology, Cat. #SC-803) at 1:5000; mouse anti-HA (Santa Cruz Biotechnology, Cat. #SC-7392) at 1:5000; goat anti-

rabbit HRP conjugate (Bio-Rad, Cat. #170-6515) at 1:3500; and goat anti-mouse HRP conjugate (Bio-Rad, Cat. #170-6516) at 1:5000.

Gels were silver stained using the Bio-Rad silver stain kit (Cat. #161-0443) according to the manufacturer's directions. Gels were stained with coomassie blue by immersion overnight in a solution containing 0.5% (w/v) coomassie brilliant blue R250, 40% (v/v) methanol, and 10% (v/v) glacial acetic acid. Gels were destained in 40% (v/v) methanol/10% (v/v) glacial acetic acid and rehydrated in distilled water.

2.1.4 Immunoprecipitations – Immunoprecipitation of YY1 was carried out using a custom-made polyclonal antibody (OpenBiosystems) raised in rabbit against a synthetic peptide with sequence AMRKHLHTHGPRVH corresponding to amino acids 268-281 of *Xenopus laevis* YY1 (Accession NP_001087404). Oocyte lysates were prepared in mRNP lysis buffer as described in section 2.1.1. All solutions and buffers used were freed of RNase by treatment with DEPC. Protein-A agarose beads (Sigma) were swollen overnight in five volumes NT2 buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.05% (v/v) Nonidet P-40) supplemented with 5% (w/v) BSA. Swollen beads were washed twice in five volumes of NT2+BSA. Antibody conjugation reactions were assembled by addition of 50 µL (10 µg) antibody to 150 µL pre-swollen protein-A agarose beads in a volume of 1 mL NT2+BSA and incubation overnight at 4 °C. Antibody conjugated beads were washed six times with 1 mL NT2+BSA and used immediately in immunoprecipitation reactions. Lysate (1 mL) was added to antibody conjugated beads and incubated with gentle agitation for 2 h at room temperature. Beads were washed six times with 1 mL NT2 (without BSA) and resuspended in 200 µL 1X SDS-PAGE loading buffer or used for extraction of mRNA as described in section 2.4.1 below.

2.1.5 Isolation of oocyte Poly-A+ RNA – Total oocyte RNA was isolated using

Trizol® extraction according to the manufacturer's direction as outlined in section 2.1.1. Isolation of poly-A+ RNA was performed in accordance with Sambrook and Russell (Sambrook, 2001). Type VII Oligo-dT cellulose (New England Biolabs) (0.5 g) was suspended in 0.1 N NaOH and poured into disposable chromatography columns. Matrices were then washed extensively with nuclease-free water and then with 1X column loading buffer (500 mM NaCl, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.1% (w/v) SDS). Samples of total RNA from 100 oocytes were suspended in 500 µL of nuclease-free water and heated at 70 °C for 5 minutes, chilled on ice, then mixed with 500 µL of 2X column loading buffer (1 M NaCl, 40 mM Tris-HCl (pH 7.6), 2 mM EDTA, 0.2% (w/v) SDS). The RNA solutions were applied to oligo-dT cellulose columns and the flow-through aliquots were collected, reheated at 70 °C, chilled, and reapplied to the column. Columns were then washed with 5 mL of 1X column loading buffer. Poly-A+ RNA was eluted by applying 2.5 mL of elution buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.05% (w/v) SDS) and collecting 200 µL fractions. The OD_{260} of the resulting fractions was measured and fractions containing RNA were pooled. Pooled fractions were supplemented with 1/10 volume 3 M sodium acetate (pH 5.2) and RNA precipitated by addition of 2.5 volumes absolute ethanol followed by centrifugation at 15,000 X g for 15 min at 4 °C. Pellets were washed once with ice-cold 75% (v/v) ethanol and resulting poly-A+ RNA fractions were resuspended in 100 µL nuclease-free water and quantified by UV spectrophotometry. Samples with OD_{260}/OD_{280} ratio of > 1.8 were utilized.

2.1.6 Size Exclusion Chromatography – Oocytes were lysed in mRNP buffer as described in section 2.1.1. Lysates were extracted once with an equal volume of 1,1,2-trichlorotriflouroethane to remove yolk proteins prior to chromatography. Lysates (300 µL) were applied to a 0.75 cm X 5 cm columns of G-200 Sephadex resin (Amersham) pre-equilibrated in a

buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 1 mM DTT. Fractions (500 µL) were collected and protein/RNA content estimated by UV spectroscopy at 260 nm and 280 nm. Fractions were precipitated by addition of four volumes of acetone followed by centrifugation at 20,000 X g for 20 min at 4 °C. Pellets were washed once with 1 mL ice-cold 80% acetone, dried, and resuspended in 50 µL 1X SDS-PAGE loading buffer containing 8 M urea. Columns were calibrated using Bio-Rad size exclusion molecular mass standard (Cat. #151-1901).

2.2 Expression and Purification of Recombinant *Xenopus laevis* YY1

2.2.1 Construction of pHA3-YY1 and pRsetB-YY1 – Plasmid isolation, transformation, DNA purification, restriction endonuclease digestion, and ligations were performed according to the protocols described by Sambrook and Russell (Sambrook, 2001), and all enzymes used were purchased from Fermentas.

The pHA3-YY1 plasmid used for expression of HA-YY1 in oocytes contains the *Xenopus* YY1 coding sequence fused to an N-terminal HA-tag under control of CMV promoter and was constructed as follows: PCR amplification was used to generate a fragment containing the *Xenopus* YY1 coding sequence from oocyte cDNA using a forward primer incorporating an *Bgl*II restriction site (5'-CGCGAGATCTATGAACATGGCATCGGGC) and a reverse primer incorporating a *Hpa*I restriction site (5'-ATATGGAAAGACATGTTATCCCAATTGGCGC). The PCR product was digested with *Bgl*II/*Hpa*I and purified by electrophoresis through low melting point agarose. The digested fragment was ligated in-frame into *Bgl*II/*Hpa*I digested, phosphatase-treated pHA3 plasmid and subsequently transformed into *E. coli* DH5α cells. The sequence of *Xenopus* YY1 was confirmed by sequencing at the Plant Biotechnology Institute, University of Saskatchewan.

pRsetBYY1, used for *in vitro* expression, contains the YY1 sequence fused to an N-terminal 6XHis tag under control of the bacteriophage T7 promoter and was constructed by sub-cloning of the YY1 coding sequence from pHA3-YY1 as follows: pHA3-YY1 was digested with *Bgl*II/*Hpa*I and the liberated fragment containing the YY1 coding sequence was isolated by electrophoresis of the endonuclease-digestion reaction through low melting point agarose. This fragment was ligated in-frame into *Bgl*II/*Pvu*II digested, phosphatase-treated pRsetB plasmid generating pRsetB-YY1. Initially, pRsetB-YY1 was maintained in *E. coli* DH5α. For expression of the YY1 protein, the pRsetB-YY1 vector was purified from cultures of DH5α cells and transformed into *E. coli* BL21(DE3) pLysS, generating the BL21(DE3) pLysS pRsetBYY1 strain.

2.2.2 Expression and Lysis – Expression of recombinant YY1 was achieved as follows: BL21(DE3) pLysS pRsetB-YY1 cells were inoculated into 3 mL aliquots of LB media (Sambrook, 2001) containing antibiotics (50 µg/mL ampicillin (Sigma) and 35 µg/mL chloroamphenicol (Sigma)) and incubated at 37 °C for 24 h. These cultures were then used to inoculate a subsequent 50 mL cultures also containing antibiotics, followed by incubation for 24 h at 37 °C. The 50 mL cultures were then used to inoculate 1 L of SOB medium (Sambrook, 2001) preheated to 37 °C. Incubation at 37 °C was continued until the OD_{600} of the culture reached 0.6 at which point it was supplemented with 2 mM IPTG and incubation continued for an additional 4 h to allow for expression of recombinant protein. Cultures were then chilled for 20 min on ice and centrifuged at 5,000 X g for 10 min at 4 °C to pellet the bacterial cells. Cells were washed once with 200 mL ice-cold STE (100 mM NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA) and collected by centrifugation at 5,000 X g for 10 min at 4 °C. Pelleted cells were lysed in 20 mL of Lysis Buffer A (8 M urea, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0), 1 % (w/v) SDS, 2

mM 2-mercaptoethanol) with vigorous shaking for 30 min, then cell lysates were sonicated 2 X 30 sec on setting five of a Sonifier® model W140 sonicator (Heat Systems-Ultrasonics Inc; Plainview, NY). Lysates were then diluted with 20 mL of Lysis Buffer B (600 mM NaCl, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0), 20 mM imidazole (pH 8.0)) and mixed thoroughly. Lysates were clarified by centrifugation at 40,000 X g for 30 min at 4 °C and stored at -80 °C.

For evaluation of different lysis buffers, equal quantities of cells suspended in STE were centrifuged (5000 X g, 5 min, 4 °C) and pellets resuspended either directly in 250 µL 1 X SDS-PAGE loading buffer (50 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 100 mM 2-mercaptoethanol, 10 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue) containing 8 M urea, or 5 mL of one of the following four lysis buffers: Lysis Buffer I (300 mM NaCl, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0), 1 X protease inhibitor cocktail (Sigma)), Lysis Buffer II (300 mM NaCl, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0), 1 X protease inhibitor cocktail (Sigma), 1 mg/mL lysozyme), Lysis Buffer III (6 M guanidium hydrochloride, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0)), or Lysis Buffer IV (8 M urea, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0), 1 % (w/v) SDS). Samples were placed on a centrifugal shaker at medium speed for 30 min then sonicated 2 X 30 sec on setting five of a Sonifier® model W140 sonicator (Heat Systems-Ultrasonics Inc; Plainview, NY). Samples were then centrifuged (40,000 X g, 30 min), and pellets resuspended in 250 µL of 1 X SDS-PAGE loading buffer containing 8 M urea. Proteins were recovered from supernatants by addition of 20 mL ice-cold ethanol and centrifuged (40,000 X g, 30 min, 4 °C) to collect precipitated protein and resulting pellets then also resuspended in 250 µL 1 X SDS-PAGE loading buffer containing 8 M urea.

Lysates for analysis of protein stability were prepared by resuspending the pelleted cells from 1 L cultures of IPTG-induced BL21(DE3) pLysS pRsetBYY1 in either 20 mL Lysis Buffer

IV, Lysis Buffer IV containing 1 mM 2-mercaptoethanol, or in Lysis Buffer IV containing 1 mM hydrogen peroxide. Suspensions were shaken at room temperature for 30 min, sonicated as above, and then diluted with 20 mL Lysis Buffer B (600 mM NaCl, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0), 20 mM imidazole (pH 8.0)), centrifuged (40,000 X g, 30 min, 4 °C), and supernatants incubated at either room temperature or 37 °C. At the indicated time points, 100 µL aliquots were removed and mixed with 25 µL of 5 X SDS-PAGE loading buffer lacking 2-mercaptoethanol, heated at 95 °C for 5 min, and analyzed by Western blotting.

2.2.3 Immobilized Metal Affinity Chromatography – Recombinant YY1 with the N-terminal 6XHis tag was purified using immobilized metal affinity chromatography (IMAC) on nickel-charged NTA-agarose (BioRad) columns. All chromatography steps were performed on a BioRad HR control unit (BioRad) at room temperature using 2 cm diameter columns with a packed volume of 10 mL resin. Prior to application of bacterial lysates, the IMAC columns were equilibrated with 50 mL Wash Buffer (300 mM NaCl, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0), 20 mM imidazole (pH 8.0), 1 mM 2-mercaptoethanol). Bacterial lysates containing recombinant YY1 (40 mL) were applied to the columns by recirculation for 16 h. Matrix was then washed with 100 mL Wash Buffer and bound protein was recovered into 5 mL fractions with 50 mL Elution Buffer (300 mM NaCl, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0), 200 mM imidazole (pH 8.0), 1 mM 2-mercaptoethanol). Fraction aliquots (100 µL) were analyzed by SDS-PAGE followed by Western blotting and Coomassie Blue staining (see 5.1.3). Fractions containing recombinant protein were assayed for protein content by the method of Bradford (Bradford, 1976), pooled, and stored at -80 °C.

Automation of column recharging procedures improved efficiency and consistency of the entire IMAC process. Columns were regenerated between uses by stripping of nickel ions from

the column with EDTA followed by acid washing, resulting in complete removal of any residual proteins from the column (Belew et al., 1987). Following elution of bound proteins, columns were regenerated as follows: Nickel ion stripping with 50 mL of 0.1 M EDTA (pH 8.0), followed by sequential washing with 100 mL water, 100 mL Acid Wash Solution (0.12 M H_3PO_4, 2 % (v/v) glacial acetic acid (pH ~2.0)), and a further 100 mL water. Columns were recharged with nickel ions by equilibration with 50 mL of 50 mM sodium acetate (pH 6.2), then subsequently with 50 mL of Nickel Solution (0.25 M $NiSO_4$, 25 mM sodium acetate (pH 6.2)), followed by a second equilibration with 50 mL of 50 mM sodium acetate (pH 6.2), and a final wash with 100 mL water. Columns were prepared for subsequent chromatography by equilibration with 50 mL Wash Buffer.

2.2.4 Renaturation – Renaturation of recombinant YY1 was carried out by spin dialysis using a Macrosep® 10K Omega (Pall Life Science) centrifugal device at 5,000 X g and 4 °C. Pooled fractions of eluted protein (see 5.2.3 above) were supplemented with guanidium isothiocyanate to a final concentration of 6 M and 0.5 M EDTA to a final concentration of 25 mM. Samples were concentrated by spin dialysis to 500 ng/µL of protein. Each step in the renaturation process was performed by two-fold diluting of preparations with Renaturation Buffer and then re-concentrating, as indicated, to 500 ng/µL. Addition of 25 mM EDTA was required to remove residual nickel ions which were found to inhibit DNA and RNA binding activity of YY1. Protein was exchanged via ten cycles of dilution and re-concentration into a Renaturation Buffer containing 150 mM KCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.2 mM $ZnCl_2$, 1 mM 2-mercaptoethanol, and 10% (v/v) glycerol. Following renaturation, recombinant YY1 was concentrated to 860 ng/µL (20 µM) and stored frozen at -80 °C until use. Samples containing different concentrations of YY1 were prepared by dilution of 20 µM stock in the

renaturation buffer described above. For some experiments, YY1 was exchanged into renaturation buffer lacking zinc and magnesium ions and instead containing 0.1 mM EDTA.

2.3 Analysis of YY1 nucleic acid binding activity

2.3.1 Radiolabelling and purification of DNA and RNA probes –

Oligonucleotide probes (See Table 1) were radiolabelled and purified as follows in accordance with the protocols of Sambrook and Russell (Sambrook, 2001). Double-stranded DNA probes were end-fill labelled using [α-^{32}P]dCTP (PerkinElmer Life Sciences) and MLuV reverse transcriptase (Fermentas). Single-stranded DNA and RNA probes were end-labelled using [γ-^{32}P]ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase (Fermentas). Double-stranded RNA probes were prepared by labelling reciprocal strands and then purified and annealed to the complimentary unlabelled strand. Double-stranded RNA probe integrity was further verified by treatment of aliquots with single-strand specific ribonuclease (data not shown). GAPDH 3'UTR and β-Globin 3'UTR probes were prepared by *in vitro* transcription of PCR products prepared as in section 2.1.2 using gene-specific primers (see Table 1) incorporating a 5' T7 promoter sequence. The Xenopus 5S rRNA was synthesized from the T7 promoter of linearized pUC19wt5S (a kind gift of P.J. Romaniuk, University of Victoria). *In vitro* transcription reactions were performed using T7 polymerase (Fermentas) and [α-^{32}P]UTP (PerkinElmer Life Sciences) by the method of Kreig and Melton (Krieg and Melton, 1984) with inclusion of 7mGpppG cap analogue (Invitrogen) in reactions generating capped transcripts. Labelled probes were diluted with 100 µL RNase-free TEN (100 mM NaCl, 20 mM Tris-HCl (pH 7.6), 2 mM EDTA) and purified by passing over 500 µL G-50 Sephadex

Table 1 – Oligonucleotides Used and Sequences Thereof

Oligo Description	Strand	Oligo Name	Sequence
YY1 Consensus DNA Element (YY1E)	F	YY1E-F	CTAGGCGCTCCCCGGCCATCTTGGCGGCTGGT
	R	YY1E-R	CTAGGACCAGCCGCCAAGATGGCCGGGGAGCG
YY1 Mutant Consensus DNA Element (YY1M)	F	YY1M-F	ACTGGCGCTCCGCGATTATCTTGGCGGCTGGT
	R	YY1M-R	ACTGGACCAGCCGCCAAGATAATCGCGGAGCG
Heat Shock Response Element DNA (HSE)	F	HSE-F	CTAGGGAAATGGAAGCCTCGGGAAACTTCGGGTCGG
	R	HSE-R	CTAGGCCGACCCGAAGTTTCCCGAGGCTTCCATTTC
GAPDH 3'-UTR	F	T7-GAPDH-F	T7-GAGAACCAGCCTCAAGAACACC
	R	GAPDH-R	TAGGAGCAGATACGAATGG
β-Globin 3'-UTR	F	T7-β-GLOBIN-F	T7-GAGAGCACTTGTCACCTGTCAAC
	R	β-GLOBIN-R	AGGAACACAGAAACAGTGAC
RNA with DNA Consensus Sequence	F	CF RNA	CGCUCCCCGGCCAUCUUGGCGGCUGGU
	R	CR RNA	ACCAGCCGCCAAGAUGGCCGGGGAGCG
RNA with Mutant DNA Consensus Sequence	F	MF RNA	CGCUCCGCGAUUAUCUUGGCGGCUGGU
	R	MR RNA	ACCAGCCGCCAAGAUAAUCGCGGAGCG
dsRNA with DNA Consensus Sequence		ds C RNA	CF RNA hybridized to CR RNA
dsRNA with Mutant DNA Consensus Sequence		ds M RNA	MF RNA hybridized to MR RNA
Poly-Uridine RNA		U_{20}	UUUUUUUUUUUUUUUUUUUU
Poly-Adenosine RNA		A_{20}	AAAAAAAAAAAAAAAAAAAA
Poly-Cytidine RNA		C_{20}	CCCCCCCCCCCCCCCCCCCC
Poly-Guanosine RNA		G_{20}	GGGGGGGGGGGGGGGGGGGG
Poly-Adenosine/Cytosine RNA		$(AC)_{10}$	ACACACACACACACACACAC
Poly-Adenosine/Guanidine RNA		$(AG)_{10}$	AGAGAGAGAGAGAGAGAGAG
Poly-Cytosine/Uridine RNA		$(CU)_{10}$	CUCUCUCUCUCUCUCUCUCU
Poly-Guanidine/Uridine RNA		$(GU)_{10}$	GUGUGUGUGUGUGUGUGU
ds Adenosine:Uridine RNA		A:U	A_{20} hybridized to U_{20}
ds Guanidine:Cytosine RNA		G:C	G_{20} hybridized to U_{20}
ds Adenosine/Uridine:Adenosine/Uridine RNA		(AU):(AU)	AUAUAUAUAUAUAUAUAUAU (Self-complementary)
ds Guanidine/Cytosine:Guanidine/Cytosine RNA		(GC):(GC)	GCGCGCGCGCGCGCGCGCGC (Self-complementary)
ds Adenosine/Cytosine:Guanidine/Uridine RNA		(AC):(GU)	$(AC)_{10}$ hybridized to $(GU)_{10}$
ds Cytosine/Uridine:Guanidine/Adenosine RNA		(CU):(AG)	$(CU)_{10}$ hybridized to $(AG)_{10}$

Table 1 – Oligonucleotides Used and Sequences Thereof (Continued)

Oligo Description	Strand	Oligo Name	Sequence
Arginine/serine-rich splicing factor 3 (ASRSF3)	F	ASRSF3-F	GCACCGCCCTATGGTTCCCG
	R	ASRSF3-R	AGGCTGGCGCTGCAGTTGTT
Arginine methyltransferase 1b (ArgMT1b)	F	ArgMT1b-F	GGGCCAGCTGTGCGAGTTGT
	R	ArgMT1b-R	GCTGCCAGCAGGGGCTGAAT
SOX3 transcription factor (SOX3)	F	SOX3-F	CCACGCGTCCGAGCACTTGT
	R	SOX3-R	CTGGGGGCTGTTCATGGCCG
Small nuclear RNP-associated protein (snRNPAP)	F	snRNPAP-F	CATTGGCAGGTGCTGCCGGA
	R	snRNPAP-R	CGCATGCCAGGTGGTGGAGG
Xenopus Nop56 (Xnop56)	F	Xnop56-F	CCGGCAGCCTCACCAACCTG
	R	Xnop56-R	CTGTGGCCTCCTGCTGTGCC
NADH dehydrogenase 1 (NADH-D-1)	F	NADH-D-1-F	CCCCGACCATTTGCAGGCGT
	R	NADH-D-1-R	GCTGCTCCAACTCAGGGCGG
Cytochrome B (Cyto-b)	F	Cyto-b-F	GGAGCCCATATTTGCCGTGACGTT
	R	Cyto-b-R	GCCGTAGTACAACCCTCGTCCG
Connexin-38 (Cnxn-38)	F	Cnxn-38-F	AGCTGGGTTCCTGCTAGGCCA
	R	Cnxn-38-R	GCATTCGGTTTCCGGCCCCA
Vitellogenin Receptor (VitR)	F	VitR-F	GGCTCTCAGGAGGGGTCGGG
	R	VitR-R	CGGCCGCTGGAGCAAGTGAA
Ribosomal Protein L3 (RPL3)	F	RPL3-F	AAGGTGGACTGGGCCCGTGA
	R	RPL3-R	CCGGGGAGTGTGCCAGAGGA

(Amersham) previously equilibrated in TEN. The resulting solution was extracted with phenol/chloroform (1:1), then chloroform/isoamyl alcohol (19:1), and precipitated by addition of 1/3 volume 7.5 M ammonium acetate and 2.5 volumes of ethanol. Oligonucleotides were collected by centrifugation at 20,000 X g for 20 min at 4 °C and pellets washed once with 1 mL ice-cold 75% ethanol. The state of purity was monitored by electrophoresis on 20% polyacrylamide, 0.5X TBE gels containing 8.3 M urea. Once no trace of unincorporated label could be detected by 12 h of radiography using Kodak XB-1 film (Kodak), oligonucleotides were quantified by UV spectroscopy in a Bio-Rad Smartspec® (BioRad) using millimolar extinction coefficients specific to the oligonucleotide being measured. Only samples with an OD_{260}/OD_{280} ratio greater than 1.8 were used.

2.3.2 YY1/DNA and YY1/RNA Electrophoretic Mobility Shift Assays – Unless otherwise specified, DNA and RNA binding reactions contained 0.1 pmol radiolabelled oligonucleotide, 50 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, and the indicated quantity of protein in a final volume of 10 µL. Reactions containing oocyte lysate, cellular lysate, or purified mRNPs, contained a final volume of 20 µL. Reactions containing RNA probes were supplemented with 10 U/µL of RNase inhibitor (Fermentas). For competition experiments, oligonucleotides (1 pmol) or antibodies (200 ng) were added directly to binding reactions. Reactions were incubated at room temperature (unless specified otherwise) for 20 min, mixed with 2 µL loading dye (0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol FF, 50% (v/v) glycerol) and immediately loaded onto non-denaturing 5% polyacrylamide gels containing 0.5 X TBE buffer (Sambrook, 2001). Gels were run at 150 V for 2.5 h at 4 °C, then dried and exposed for 16 h to XB-1 film (Kodak) at -80 °C with an intensifying screen.

2.3.3 Nitrocellulose Filter Binding Assays – Nitrocellulose filter binding assays

were performed according to Romaniuk (Romaniuk, 1985) with slight modifications. Binding reactions were carried out by addition of various concentrations of recombinant YY1 to 0.5 pmol radiolabelled probe in a final volume of 50 µL of Binding Buffer (50 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$ and 1 mM DTT). For binding reactions using RNA probes, binding reactions were supplemented with 50 U RNase-inhibitor (Fermentas). After 20 min at room temperature, reactions were diluted with 50 µL Binding Buffer and immediately applied to pre-equilibrated 0.45 µm nitrocellulose membranes (Bio-Rad) contained in a Bio-Rad dot-blot apparatus. Filters were washed twice with 100 µL binding buffer and then dried for 90 min at 80 °C *in vacuo*. Filters were dissolved in 500 µL benzethonium hydroxide (Alfa Asar) in methanol then mixed with 15 mL of scintillation fluid. Radioactivity was measured in a Tri-Carb 3100TR scintillation counter (Packard Biosciences, Meriden, CT). Background readings were subtracted from all data values and K_d values were calculated using the MatLab® software package. Presented data is the average of three replicate experiments.

2.3.4 Determination of YY1 minimal binding site – U_{20} RNA was partially nuclease-digested by combining 5 pmol end-labelled (see section 2.3.1) U_{20} RNA with 1 ng RNase A (Fermentas) in 50 µL of RNase-free TMN (100 mM NaCl, 20 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$) followed by incubation at room temperature for 20 minutes. Reactions were quenched by addition of 0.5 M EDTA (pH 8.0) to a final concentration of 5 mM, and then extracted twice with 1:1 (v/v) phenol/chloroform and then once with 19:1 (v/v) chloroform/isoamyl alcohol. The aqueous phase was then supplemented with 25 µL 7.5 M ammonium acetate and 10 µg RNA-grade glycogen (Fermentas) and the RNA precipitated by addition of 2.5 volumes absolute ethanol. After chilling on ice (10 min) the precipitated RNA samples were collected by centrifugation (20,000 x g, 20 min, 4 °C) and washed once with 250

µL of ice-cold 80% (v/v) ethanol. The dry pellet was used immediately in YY1 RNA-binding reactions.

RNA binding reactions were assembled by resuspension of digested RNA probe in 200 µL RNA-binding buffer (50 mM NaCl, 50 mM NaH_2PO_4/Na_2HPO_4 (pH 7.5), 2 mM MgCl2, 0.5 mM 2-mercaptoethanol, and 0.1 U/µL RNase-inhibitor (Fermentas)) and adding 0.2 nmol recombinant YY1. Control reactions were identical except no recombinant YY1 was added. Binding reactions were incubated at room temperature for 20 min and then transferred to a fresh tubes containing 50 µL nickel-charged NTA-agarose resin (BioRad) pre-equilibrated with binding buffer. Reactions were incubated a further 20 min at room temperature with gentle rotation. NTA-agarose beads were collected by centrifugation (1000 x g, 1 min) and washed with 3 X 1 mL RNA-binding buffer. Washed beads were resuspended in 50 µL formamide loading buffer (80% (v/v) formamide, 100 mM Tris-HCl (pH 8.0), 0.05 % (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF) and heated at 95 °C for 5 min then chilled on ice. NTA-agarose beads were removed by centrifugation (12,000 x g, 10 min) and supernatants analyzed immediately by denaturing polyacrylamide gel electrophoresis.

For resolution of small RNA fragments a special high-strength acrylamide gel solution containing a 20 % (w/w) acrylamide, 12:1 acrylamide:bisacrylamide ratio was prepared as follows: 49.8 g urea (8.3 M final concentration, Sigma), 18.4 g acrylamide (Sigma), 1.6 g bisacrylamide (Sigma), and 10 mL 5 X TBE Buffer (0.22 M Tris Base, 0.22 M Boric Acid, 5 mM EDTA) were dissolved in water to a final volume of 100 mL. Gel solutions were stored at room temperature and used within 2 - 3 weeks of preparation. To prepare gels, 25 mL of the above acrylamide solution was mixed with 250 µL of 10% (w/v) ammonium persulfate and 10 µL of N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED) and poured at a thickness of 0.75 mm. All

electrophoresis steps were performed using 0.5 X TBE as the tank buffer. Prior to loading of samples, gels were pre-run at 200 V for 20 min. Analysis of RNA-binding reactions was performed by loading approximately 20 cps of RNase-digested RNA probe (as marker) and 5 µL of formamide eluant obtained from RNA-binding reactions described above into the adjacent lanes. Electrophoresis was conducted at 200 V for approximately 1.5 hours, until the bromophenol blue fronts had migrated approximately 2/3 of the distance to the bottom of the gel. Following electrophoresis, gels were removed, covered with plastic wrap, and exposed to Kodak XB-1 radiography film with an intensifying screen at -80 °C for periods varying from 12 h to 10 days.

2.3.5 Oligo-dT Cellulose Chromatography and Purification of YY1 from Oocyte mRNPs – For preparation of oocyte mRNPs by oligo-dT cellulose chromatography, lysates prepared as in section 2.1.1 were extracted with an equal volume of 1,1,2-trichlorotrioflouroethane to remove yolk proteins and supplemented with additional sodium chloride to a final concentration of 300 mM NaCl. Cleared lysate (1 mL) was applied to 100 mg of pre-equilibrated type VII Oligo-dT cellulose (New England Biolabs) and incubated with gentle agitation for 2 h at 4 °C. Following incubation matrix mixtures were washed twice with five volumes ice-cold Binding Buffer (300 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT). For isolation of native mRNPs, mixtures were batch-eluted twice with 2 mL of Elution Buffer (300 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 30% (v/v) de-ionized formamide). Eluted mRNPs were exchanged into Renaturation Buffer (50 mM KCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.2 mM $ZnCl_2$, 1 mM DTT) and concentrated to a volume of 0.5 mL by spin-dialysis using a Microsep 10K omega centrifugal device (Pall Corporation) yielding approximately one oocyte equivalent of mRNPs per 10 µL. For analysis of HA-YY1

expressing oocytes, matrix mixtures were eluted directly in 200 µL of 1X SDS-PAGE loading buffer. For analysis of recombinant YY1, binding reactions were carried out in the presence of 100 mg of oligo-dT cellulose, 0.5 µg of poly-A+ oocyte RNA (see section 2.1.5) and 10 pmol of rYY1 and either 1 µg RNase A (Fermentas) or 20 pmol of U_{20} or C_{20} as indicated.

Analysis of the stability of mRNP-associated YY1 with respect to ionic perturbation was conducted using oligo-dT cellulose chromatography as follows: lysates were freed of yolk proteins by extraction of 1,1,2-trichlorotriflouroethane as described above. Columns were maintained at 4 °C and run at a flow-rate of 0.1 mL/min using a BioLogic HR control unit (BioRad). Lysates (1 mL) were applied to type VII oligo-dT cellulose (New England Biolabs) columns with a bed volume of 2 mL after pre-equilibration in Binding Buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT). Bound proteins were eluted in 200 µL fractions with the above binding buffer containing a range of NaCl concentrations from 100 mM to 2 M. Proteins were precipitated by adding 4 volumes of ice-cold acetone and pelleted by centrifugation at 20,000 X g for 20 min at 4 °C. Precipitated proteins were resuspended in 50 µL 1X SDS-PAGE loading buffer and analyzed by Western blotting as described in section 2.1.3.

2.4 Immunoprecipitation and Identification of YY1-associated mRNA by DNA Microarray

2.4.1 Isolation of Immunoprecipitated mRNA - Following immunoprecipitation with anti-YY1 antibody as described above (see 2.1.4), protein-A agarose beads were suspended in 1 mL Trizol(R) (Invitrogen) reagent. RNA was isolated from immunoprecipitated material as follows: samples suspended in Trizol were supplemented with 200 µL of chloroform and vortexed vigorously then centrifuged at 12,000 x g for 10 min at 4 °C. Supernatants were collected and extracted with 600 µL of 19:1 (v/v) chloroform/isoamyl alcohol and centrifuged

(12,000 X g, 5 min, 4 °C). Supernatants were collected and RNA precipitated by the addition of 10 µg RNase-free glycogen (Fermentas) as carrier and 500 µL isopropanol. After chilling on ice (10 min) RNA was collected by centrifugation at 20,000 x g for 20 min at 4 °C. Pellets were rinsed once with 1 mL 75% (v/v) ethanol. After allowing excess ethanol to evaporate, RNA samples were resuspended in 50 µL RNase-free water and RNA quality was determined non-destructively by UV spectroscopy in sterile, RNase-free methacrylate cuvettes (BioRad). Following spectroscopic analysis, 5.5 µL of 3 M Sodium Acetate (pH 5.2) and 140 µL absolute ethanol were added and RNA samples were stored as the ethanol precipitate at -20 °C until use.

2.4.2 First-cycle Reverse Transcription - RNA samples stored as the ethanol precipitates were centrifuged (20,000 x g, 20 min, 4 °C) and pellets dried briefly to remove excess ethanol. The dried pellets were then resuspended in 3.0 µL RNase-free water then mixed with 1.0 µL "poly-A spike-in control RNA" (Affymetrix) and 1.0 µL of 5 uM Oligo-dT/T7 primer were added ("poly-A spike-in control RNA" contains a mixture of bacterially derived transcripts at known concentrations, these transcripts are used to normalize signal intensity values during reading of the microarray). Mixtures were heated at 70 °C for 10 min and then chilled on ice for 5 min. Reverse transcription reactions were assembled by addition of 2.0 µL 5X First Strand Buffer (Invitrogen), 1.0 µL of 0.1 M DTT, 0.5 µL (20 U) of RNase-inhibitor (Fermentas), 0.5 µL of 10 mM dNTPs, and 1.0 µL of SuperScript Reverse Transcriptase (Invitrogen). Reactions were incubated at 42 °C for 1 h then heated at 70 °C for 10 min to inactivate Reverse Transcriptase, then chilled on ice. Second-strand synthesis reactions were assembled by addition of the following to the chilled first-strand reaction (10 µL): 4.8 µL RNase-free water, 4.0 µL of 17.5 mM MgCl2, 0.4 µL of 10 mM dNTP mix, 0.6 µL E. coli DNA polymerase I (Invitrogen) and 0.2 µL RNase H (Invitrogen). Reactions were incubated at 16 °C

for 2 hours then heated to 75 °C for 10 min and chilled on ice.

2.4.3 *In Vitro* Transcription - The double-stranded cDNA solutions obtained above were used directly in *in vitro* transcription reactions using the Ambion MegaScript T7 *in vitro* transcription kit (Ambion) components with modification of the supplier's protocol as follows: To cDNA solutions (20 µL) were supplemented with 5.0 µL 10X IVT buffer, 5.0 µL 75 mM ATP, 5.0 µL 75 mM GTP, 5.0 µL 75 mM CTP, 5.0 µL 75 mM UTP, and 5.0 µL T7 RNA Polymerase giving a final volume of 50 µL. Reactions were incubated at 37 °C for 16 h then chilled on ice. *In vitro* transcribed RNA (cRNA) was purified and separated from template DNA via the Affymetrix cRNA cleanup kit (Affymetrix) according to the manufacturer's direction. The purified cRNA was used immediately in second-cycle cDNA synthesis as described below.

2.4.4 Second-cycle Reverse Transcription – The cRNA solutions obtained above in a volume of 9.0 µL were mixed 2.0 µL of 200 ng/µL random primers (Invitrogen), heated at 70 °C for 10 min, chilled on ice (5 min) and mixed with: 4.0 µL 5X First Strand Buffer (Invitrogen), 2.0 µL 0.1 M DTT, 1.0 µL (40 U) RNase-inhibitor (Fermentas), 1.0 µL of 10 mM dNTP mix, and 1.0 µL SuperScript Reverse Transcriptase (Invitrogen) for a final volume of 20 µL. Reactions were incubated at 42 °C for 1 h, chilled briefly on ice, supplemented with 1.0 µL RNase H (Invitrogen), and incubated a further 20 min at 37 °C. The reaction was then heated at 95 °C for 5 min to inactivate enzymes and chilled on ice. Second strand reactions were assembled by addition of 4.0 µL of 5 µM Oligo-dT/T7 primer followed by heating at 70 °C for 10 min, chilling on ice (5 min), and addition of 88 µL RNase-free water, 30 µL 5X Second Strand Buffer (Invitrogen), 3.0 µL 10 mM dNTP mix, and 4.0 µL of E. coli DNA Polymerase I (Invitrogen) to final volume of 150 µL. Second-strand reactions were incubated for 2 h at 16 °C, supplemented with 2.0 µL T4 DNA Polymerase (Fermentas) and incubation at 16 °C for 10 min

and then chilled on ice. cDNA samples were purified using the Affymetrix cDNA Cleanup Kit (Affymetrix) according to the manufacturer's direction.

2.4.5 Preparation of Double-Stranded cDNA from Oocyte Total RNA –

Synthesis of double-stranded cDNA incorporating a T7 promoter from total oocyte RNA was achieved similarly to the first-cycle reverse transcription of immunoprecipitated mRNA discussed in section 2.4.2 with some modifications. RNA samples stored as the ethanol precipitate were centrifuged (20,000 X g, 20 min, 4 °C), and pellets were dried briefly to remove excess ethanol and resuspended in nuclease-free water. RNA concentrations were determined by UV spectrophotometry, sample volumes adjusted to 5 µg total oocyte RNA per 10 µL, and combined with 2.0 µL "poly-A spike-in control RNA" (Affymetrix) and 2.0 µL of 50 µM Oligo-dT/T7 primer. The mixture was heated at 70 °C for 10 min and then chilled on ice for 5 min. Reverse transcription reactions were assembled by addition of 4.0 µL 5X First Strand Buffer (Invitrogen), 2.0 µL of 0.1 M DTT, 1.0 µL (40 U) of RNase inhibitor (Fermentas), 1.0 µL of 10 mM dNTPs, and 2.0 µL of SuperScript Reverse Transcriptase (Invitrogen) for a final volume of 20 µL. Reactions were incubated at 25 °C for 10 min, 42 °C for 1 h, then heated at 70 °C for 10 min to inactivate Reverse Transcriptase, and chilled on ice. Second strand synthesis reactions were assembled by addition of the following to the chilled first strand reaction (20 µL): 9.6 µL RNase-free water, 8.0 µL of 17.5 mM MgCl2, 0.8 µL of 10 mM dNTP mix, 1.2 µL E. coli DNA Polymerase I (Invitrogen) and 0.4 µL RNase H (Invitrogen). Reactions were incubated at 16 °C for 2 h, supplemented with 2.0 µL T4 DNA Polymerase (Invitrogen), incubated for an additional 10 min at 16 °C, then heated to 75 °C for 10 min and chilled on ice. cDNA samples were purified using the Affymetrix cDNA Cleanup Kit (Affymetrix) according to the manufacturer's direction.

2.4.6 Synthesis of Biotinylated RNA and DNA Microarray Analysis - Production of biotinylated cRNA for gene chip analysis was performed using the Affymetrix Gene Chip IVT Labelling Kit (Affymetrix) according to the manufacturer's directions. Briefly, all the amplified cDNA obtained from either the two-cycle amplification procedure or the single-cycle procedure used with total oocyte mRNA described above was combined with 4.0 µL 10X IVT Reaction Buffer, 12 µL IVT Labelling NTP Mix, and 4.0 µL IVT Enzyme Mix and RNase-free water to make a final volume of 40 µL. *In vitro* transcription reactions were incubated at 37 °C for 16 h. The resulting biotinylated cRNA samples were purified using the Affymetrix cRNA Purification Kit (Affymetrix) according to the supplied directions. Aliquots were diluted 1/50 in RNase-free water and RNA quantified by UV spectrophotometry using a BioRad SmartSpec Plus spectrophotometer (BioRad) and a conversion ratio of 40 µg ssRNA per OD unit at 260 nm. All samples exceeded the minimum OD_{260}/OD_{280} ratio of 1.8 required for gene chip analysis. Integrity and quantity of *in vitro*-transcribed cRNA was verified by electrophoresis on 1% agarose-TAE gels containing 0.5 µg/mL ethidium bromide. Microarray analysis was performed by Genome Explorations Inc, Memphis, TN using the Affymetrix *Xenopus* Genome 2.0 oligonucleotide microarray.

2.4.7 Treatment of DNA Microarray Data – Computerized data manipulations were performed by Mr. Justin Guenther using MATLAB® software package and software written in the Perl programming language (Lab of Dr. N. Ovsenek, Department of Anatomy and Cell Biology, University of Saskatchewan) in accordance with the published literature (Vallee et al., 2008). For total mRNA analysis, control (total) mRNA from five replicate females, and YY1 IP mRNA from three replicate females were analyzed by DNA microarray. Normalized signal intensities from each replicate array were averaged to generate the Control and YY1 IP data sets.

Data was divided into two categories: Positives (mRNAs predicted to associate with YY1) and Negatives (mRNAs predicted not to associate with YY1 but present in the oocyte pool). Positive results encompassed spots that had log signal intensity > 3 in the Control data set and > 3.17 in the YY1 IP data set. Negative results encompassed spots that had log signal intensity > 3 in the Control data set and < 2.7 in the YY1 IP data set. Gene sequences of *Xenopus laevis* are not currently annotated with Gene Ontology (GO) terms. Therefore, in order to associate functions with the products of mRNAs bound by YY1 in oocytes, it was necessary to align the sequences of mRNAs detected in YY1 IPs with the more fully annotated *Mus musculus* nucleotide sequence database. *Xenopus laevis* sequences were aligned with the NR database at NCBI. Protein accession numbers in the UniProt database were retrieved for BLAST hits with alignment expectation values to *Mus musculus* less than 1×10^{-20}. GO terms associated with the highest scoring BLAST hit for each sequence were tallied from annotation data in the UniProt database.

2.4.8 Verification of Gene Chip data by Real Time PCR – For Real Time PCR analysis, mRNA was isolated from immunoprecipitation reactions as described above (2.4.1). Reactions containing five oocyte equivalents immunoprecipitated mRNA or total oocyte RNA, and 0.5 µg Oligo-dT(18) primer in a volume of 10 µL RNase-free water were heated at 70 °C for 10 min and then chilled on ice. Reverse transcription reactions were assembled by adding 2 µL 10X RT buffer (Fermentas), 200 µM dNTPs, 40 U RNase inhibitor (Fermentas), and 200 U MLuV H- Reverse Transcriptase (Fermentas) in a final volume of 20 µL and incubated at 42 °C for 1 h. Reverse transcriptase reactions were terminated by heating at 70 °C for 10 min followed by chilling on ice. The resulting cDNA was diluted with 30 µL water and one microliter of the resulting cDNA was used in each PCR reaction. PCR reactions contained 1 X SYBR green/Taq

Polymerase master mix (Fermentas), 0.2 µM forward and reverse primer, 0.2 mM dNTPs, and 0.1 oocyte equivalent cDNA. Reactions were conducted using a Bio-Rad iCycler® (Bio-Rad) quantitative PCR instrument. Data was analyzed according to Pfaffl *et al.* (Pfaffl, 2001) using the Bio-Rad CFX Manager® software package (Bio-Rad). Presented data is the average of three replicates using starting RNA from oocytes of three separate females.

2.5 Analysis of Nucleocytoplasmic distribution of YY1 in cultured cells

2.5.1 Cell lines, culture conditions, and preparation of lysates – Human hepatocellular carcinoma HepG2 cells were cultured in DMEM media (Sigma) containing 10% (v/v) foetal bovine serum (Sigma) and supplemented with penicillin/streptomycin. *Xenopus* A6 kidney epithelial cells were cultured at 26 °C in 85% (v/v) MEM containing 10% (v/v) foetal bovine serum and supplemented with penicillin/streptomycin. Whole cell extracts were prepared by homogenization in 400 mM NaCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 10 % (v/v) glycerol, 1X protease inhibitor cocktail (Sigma) followed by centrifugation at 15,000 X g for 15 min at 4 °C. Nuclear and cytoplasmic extracts were prepared by a modification of the method of Dignam *et al.* (Dignam et al., 1983). Cells were harvested by trypsinization and collected by centrifugation (800 X g, 5 min, 4 °C) and were washed once in phosphate buffered saline. Cell pellets were gently resuspended in 500 µL buffer A (10 mM KCl, 10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 0.5% (v/v) triton X-100, 1X protease inhibitor cocktail (Sigma)) and agitated by end-over-end rotation at 4 °C for 10 min. Nuclei were collected by centrifugation (1000 X g, 5 min, 4 °C) and the supernatants containing the cytoplasmic fractions removed and saved. The cytoplasmic fraction was then mixed with 60 µL of buffer B (1.4 M KCl, 300 mM HEPES (pH 7.9)) and 50 µL glycerol, vortexed vigorously, and centrifuged at 15,000 X g for 15 min at 4 °C. The supernatants, comprising the final cytoplasmic extracts, were

stored at -80 °C until use. The pelleted nuclei were washed by gentle resuspension in buffer A followed by a second centrifugation at 1000 X g for 5 min at 4 °C. Washed nuclei were then suspended in 250 µL Buffer C (400 mM NaCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 10 % (v/v) glycerol, 1X protease inhibitor cocktail) and homogenized with ten strokes of a Dounce homogenizer. Homogenates were centrifuged (15,000 X g, 15 min, 4 °C) and the supernatants stored at -80 °C until use.

2.5.2 EMSA analysis of YY1 DNA- and RNA- binding Activity in Culture Cell Lysates – EMSA analysis of YY1 DNA- and RNA-binding activity in lysates of cultured cells was performed essentially as described in section 2.3.2. Protein concentrations in lysates were determined by the method of Bradford (Bradford, 1976) and concentrations were adjusted to 4 µg/µL. In contrast to binding reactions using recombinant YY1, binding reactions using cell lysates contained 0.5 µg poly-dIdC (GE Life Science) and 20 µg protein (5 µL lysate) in a final volume of 20 µL.

3. Results

3.1 Purification and Renaturation of Recombinant YY1 - The initial sections of the results describe parameters for effective *in vitro* production of YY1, a zinc finger nucleic acid-binding protein with structural features requiring optimization of purification and renaturation protocols.

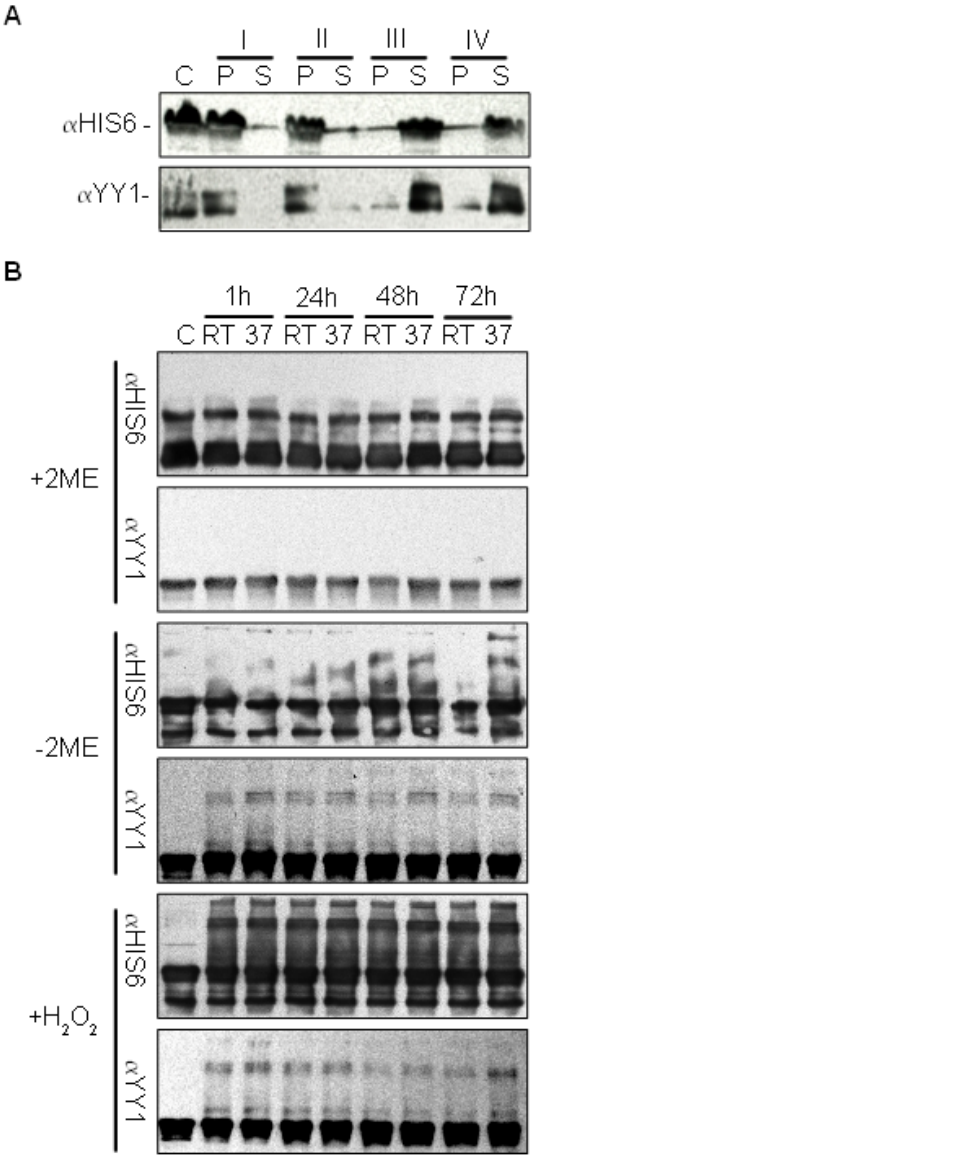
3.1.1 Lysis Buffer Efficiency – YY1-expressing bacteria were lysed in four different lysis buffers, and both soluble and insoluble fractions were analyzed by Western blotting (Figure 2A). In controls, sonication of cells suspended in simple buffers lacking lysis reagents resulted in the majority of recombinant protein remaining in the pellet (Figure 2A). Addition of lysozyme did not result in a substantial increase in levels of recombinant protein in the soluble fraction (Figure 2A), despite verification of the activity of this lysozyme according to Zinder *et al.* (Zinder and Arndt, 1956) (data not shown). The majority of recombinant YY1 was present in the soluble fraction with lysis buffers containing either 6 M guanidium hydrochloride or 8 M urea.

3.1.2 Effect of Reducing Agents on YY1 in Bacterial Lysates – In relatively concentrated protein solutions under non-denaturing conditions proteins are susceptible to formation of intermolecular as well as intramolecular disulfide bonds (Andersson, 1969; Andersson, 1970). This issue was examined with respect to YY1 by analysis of lysates prepared in the absence or presence of either 2-mercaptoethanol or hydrogen peroxide. Oxidation-dependent aggregation was assessed by removing aliquots at the indicated time points and analysis by Western blotting under non-reducing conditions. Non-reducing gels were used in order to detect aggregated YY1 that could potentially be formed in lysates lacking reducing agents (Andersson, 1969; Andersson, 1970). No significant degradation products or higher

molecular weight aggregates of recombinant YY1 were detected in the presence of 2-mercaptoethanol (Figure 2B, upper two panels), whereas high molecular mass aggregates were detected after as little as 1 h of room temperature incubation in the absence of 2-mercaptoethanol (Figure 2B, middle two panels). Formation of aggregates was shown to be dependent on protein oxidation since supplementation of buffer with the oxidising agent hydrogen peroxide resulted in similar aggregation of YY1 after 1 h (Figure 2B, lower two panels). It is hypothesized that oxidation of key cysteine residues responsible for co-ordination of zinc ions in the zinc-finger regions of the protein could account for the sensitivity of YY1 to oxidation. In no case were lower molecular mass bands detected that would suggest proteolytic degradation of recombinant YY1 under these conditions.

<u>Figure 2</u> – Effect of Lysis Buffer Composition on YY1 Solubility and Stability. (A) YY1-expressing *E. coli* were lysed directly in SDS-PAGE loading buffer containing 8 M urea (control, C) or in Lysis Buffers I – IV as indicated above the panel (see materials and methods for buffer compositions). Lysis Buffer I contained 300 mM NaCl, Buffer II was 300 mM NaCl + Lysozyme, Buffer III contained 6 M guanidine hydrochloride, and Buffer IV contained 8 M urea + 1 % SDS. Insoluble (P, pellet) and soluble (S, supernatant) fractions were separated by centrifugation and analyzed by Western blotting using anti-HIS6 tag (αHIS6) or anti-YY1 (αYY1) antibodies. (B) Effect of reducing agents on YY1 aggregation in lysates prepared in Lysis buffer IV were analyzed by Western blotting following electrophoresis on non-denaturing SDS-PAGE gels. Lysates were prepared in the presence (+2ME) or absence (-2ME) of 2-mercaptoethanol or in the presence of hydrogen peroxide (+H_2O_2). Temperature and duration of incubation in hours is indicated above the panel. (RT, room temperature; 37 °C). Control lysates (C) were prepared directly in SDS-PAGE loading buffer containing 8 M urea without 2-mercaptoethanol.

Figure 2


A
I
II
III
IV
C P S P S P S P S
αHIS6 -
αYY1-
B
1h
24h
48h
72h
C RT 37 RT 37 RT 37 RT 37
+2ME
-2ME
+H2O2
αHIS6
αYY1

3.1.3 Immobilized Metal Affinity Chromatography of YY1 – Lysates in buffer containing 4 M urea, 300 mM NaCl, 50 mM Phosphate buffer, 0.5 % SDS and 2-mercaptoethanol were applied to nickel-charged NTA-agarose columns by recirculation. It was determined empirically that increased purity of recombinant protein could be achieved using wash buffers with higher concentrations of imidazole than those recommended by the manufacturer. Inclusion of 20 mM imidazole in Wash Buffers resulted in eluted protein with >95 % purity as compared to approximately 80-90 % purity with standard wash buffers (data not shown). The use of 4-8 M urea in lysis and wash buffers is purported to enhance the specificity of binding of HIS-tagged proteins to metal-charged affinity columns (Esfandiar et al., 2010; Zhu et al., 2005). Under these conditions, metal ion-binding proteins of bacterial origin are denatured while the HIS-tag moiety retains the capacity to complex with immobilized metal ions on the matrix. Initial experiments with YY1 isolation employed wash buffers containing 4 M urea. However, due to the large quantities of urea required (960 g for a 4 L carboy of buffer) the efficacy of wash buffers lacking denaturants was examined further. Whereas urea enhanced the specificity of recombinant YY1 binding to columns in load-on procedures when included in lysis buffers, it did not significantly enhance purity and yield when included in wash buffers (data not shown). Contrarily, adjustment of imidazole concentrations in wash buffers had the greatest effect on purity of recombinant protein eluted from the column (data not shown). Use of guanidium hydrochloride as a denaturant in wash and elution steps resulted in significant leakage of nickel ions from the column matrix as determined by spectroscopic analysis (data not shown), the impact of which is discussed in section 3.1.4 below.

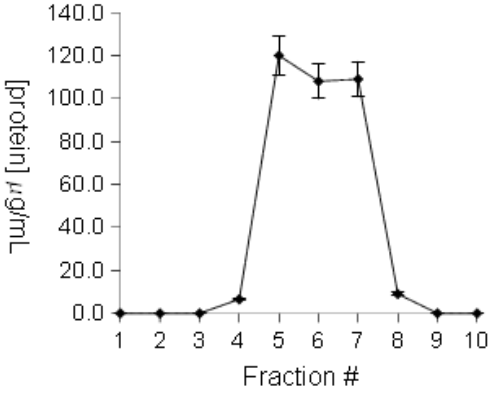
A systematic comparison of elution buffers demonstrated that imidazole was superior to pH elution using either acetate or 2-(N-morpholino)ethanesulfonic acid (MES)

buffers. In the case of YY1, pH elution resulted in precipitation near its isoelectric point (pH 5.8). Whilst not applicable to YY1, pH elution remains highly desirable in many applications since it obviates removal of imidazole which is a potential inhibitor of certain protein functions. Elution of YY1 into ten 5 mL fractions was performed with buffer containing 200 mM imidazole (pH 8.0). Rather than bulk elution, selection of fractions containing peak concentrations of protein facilitated more concentrated final protein product, and this practice eliminated time consuming protein concentration steps. While a small quantity of protein was observed to elute into early fractions (Figure 3B) the majority of recombinant protein eluted into fraction numbers 5-8 (Figure 3). Western blotting showed that fraction 7 contained the bulk of eluted YY1 protein (Figure 3B) and this fraction was used for subsequent renaturation.

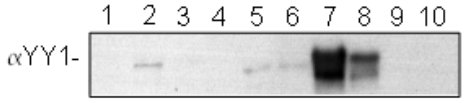
Figure 3 – Imidazole-Elution of YY1 from IMAC Resins. (A) Ten 5 mL fractions were collected during elution of YY1-loaded IMAC columns with 200 mM imidazole for determination of protein concentrations. Fraction numbers are plotted on the x-axis, protein concentration in μg/mL is plotted on the y-axis. (B) Fractions were analyzed by Western blotting using anti-YY1 antibody (αYY1). Fraction numbers are indicated above the panel.

Figure 3

A


140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
[protein] μg/mL
1 2 3 4 5 6 7 8 9 10
Fraction #

B


1 2 3 4 5 6 7 8 9 10
αYY1-

3.1.4 Renaturation of YY1 – An empirical approach to achieving optimal activity of recombinant YY1 showed the need for removal of nickel ion contamination in eluants. Optimal results were obtained through a procedure involving initial denaturation of eluted protein with simultaneous chelation of nickel and other potential contaminants, followed by renaturation.

Column fractions containing YY1 were supplemented with solid guanidium isothiocyanate (as a denaturant) to a final concentration of 6 M and EDTA to a final concentration of 25 mM. Addition of 6 M guanidium isothiocyanate resulted in effective denaturation of YY1 as demonstrated by diagnostic DNA EMSA analysis of aliquots of guanidium supplemented fractions (Figure 4B). Samples were supplemented with EDTA in order to complex residual nickel ions from the IMAC step that inhibit YY1 nucleic acid binding activity (Figure 4C). YY1 was renatured by stepwise buffer exchange using a centrifugal dialysis device. Progressive renaturation of YY1 though stepwise buffer exchange was monitored by nitrocellulose filter binding assays and EMSA analysis. As determined by these assays, YY1 renatured as the guanidium concentration was reduced from 750 mM to 94 mM with no further increases in activity noted below 94 mM (Figure 4A and B).

Nickel-charged NTA-agarose generally exhibits low leakage of nickel ions into solution at pH 8.0 (Belew et al., 1987). Free nickel at low concentrations could conceivably bind to proteins possessing zinc-fingers, potentially inhibiting activity of target proteins. Indeed, spectroscopic analysis of eluants revealed the presence of the Ni^{2+} ion (data not shown). Addition of 1 mM nickel sulphate to binding reactions containing YY1 significantly decreased its activity (Figure 4C) demonstrating that the presence of relatively small amounts of nickel ions inherent to the IMAC procedure can inhibit the DNA-binding activity of YY1 *in vitro*, presumably through inhibition of zinc-finger function. Addition of EDTA to eluants prior to renaturation was

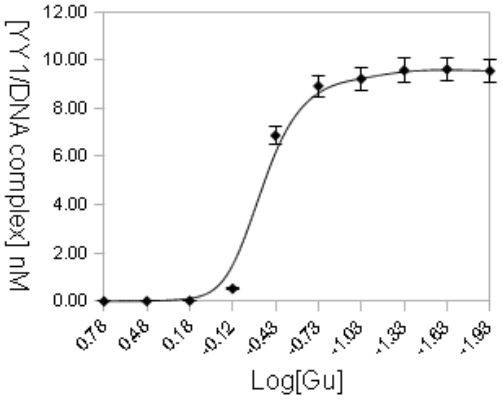
necessary to attain full activity of recombinant YY1 protein, suggesting this step effectively removes bound nickel from recombinant YY1.

The importance of maintaining reducing conditions during the isolation and renaturation of YY1 is demonstrated by the experiments in Figure 4C. Renaturation of YY1 in the absence of 2-mercaptoethanol resulted in significantly lower activity compared to renaturation under mild reducing conditions (Figure 4C). Subsequent addition of hydrogen peroxide to active YY1 resulted in a decline in DNA-binding activity to a level that was barely detectable by EMSA (Figure 4C). Collectively these data demonstrate the importance of considering redox and metal ion effects on YY1 activity.

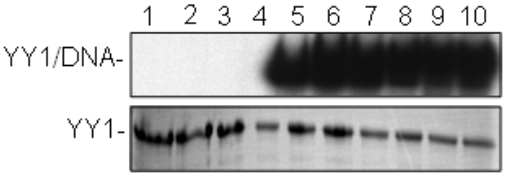
<u>Figure 4</u> – Renaturation of Recombinant YY1. YY1 was renatured through stepwise (10) exchange of buffer starting from 6 M guanidium isothiocyanate with 50% dilution of guanidium at each step. (A) Aliquots of YY1 from each step were analyzed for DNA-binding activity by nitrocellulose filter binding assays containing 10 nM labeled YY1 DNA element. The logarithm of guanidium isothiocyanate concentration at each step of buffer exchange is plotted on the x-axis. Concentration of YY1-DNA complex formed in each aliquot is plotted on the y-axis. (B) Renaturation of YY 1 was analyzed by EMSA using labeled YY1 DNA element (upper panel). Presence of YY1 in the analyzed fractions is demonstrated by SDS-PAGE of aliquots followed by staining with Coomassie Blue (lower panel). (C) Effect of redox conditions and free nickel on YY1 DNA-binding activity assayed by EMSA. The DNA-binding activity of YY1 renatured under ideal conditions (control, C) is compared to the activity of YY1 supplemented with 1 mM hydrogen peroxide ($+H_2O_2$), renatured in the absence of 2-mercaptoethanol (-2ME), or supplemented with 1 mM nickel sulfate ($+NiSO_4$).

Figure 4

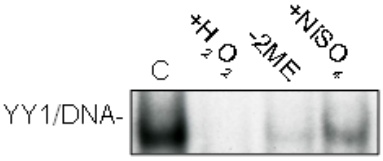
A


12.00
10.00
8.00
6.00
4.00
2.00
0.00
[YY1/DNA complex] nM
0.78
0.48
0.18
-0.12
-0.43
-0.73
-1.03
-1.33
-1.63
-1.93
Log[Gu]

B


1 2 3 4 5 6 7 8 9 10
YY1/DNA-
YY1-

C


C
+H2O2
-2ME
+NISO4
YY1/DNA-

3.2 YY1 Possesses Sequence-Specific RNA Binding Activity

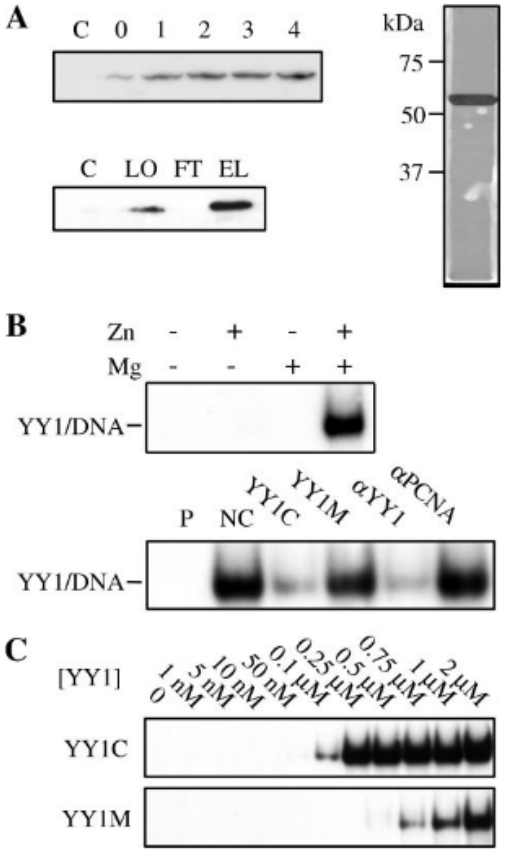
3.2.1 Analysis of Recombinant YY1 – Following bacterial expression and purification of His_6-tagged *Xenopus* YY1 (Figure 5A), activity of the recombinant protein was verified by DNA-binding assays using the consensus promoter element. YY1 required the presence of both zinc and magnesium ions for DNA binding (Figure 5B). Empirical experimentation revealed that, at minimum, concentrations of 1 mM magnesium and 50 μM zinc were required for activity. Subsequently, the renaturation buffer used was standardized at concentrations of 2 mM magnesium and 0.2 mM zinc respectively. Formation of YY1-DNA complexes was competed with both oligonucleotide and antibody competitors (Figure 5B) in a manner similar to that of endogenous YY1 (Ficzycz et al., 2001). Recombinant YY1 bound with high affinity to the consensus YY1-DNA element and with considerably less affinity to a mutated consensus DNA probe (Figure 5C), as observed in previous studies using mammalian YY1 (Momoeda et al., 1995; Yant et al., 1995). No binding activity was observed with single-stranded forward and reverse strands of either consensus or mutant DNA probes (data not shown). Therefore, YY1 did not possess affinity for single-stranded DNA.

Nitrocellulose filter binding reactions were used for comparative determination of dissociation constants of various probes and to quantitatively verify the results of the EMSA analysis. The filter binding assay measures the proportion of the total radioactivity bound, which is converted to a molar quantity of bound probe as a function of the specific activity. High affinity of YY1 for consensus DNA probe was verified by filter binding assay ($K_d = 3.2 \pm 0.1$ nM, Table 2). Moderate to low affinity binding to mutant probe ($K_d = 54.9 \pm 1.2$ nM) and no detectable binding to heat shock element DNA were observed (Figure 5C and Table 2). Experiments employing modified EMSA protocols showed that YY1 did not exhibit free

nucleotide binding activity to adenosine, uridine, or deoxycytidine triphosphates (data not shown).

Figure 5 – Purity and DNA-binding Activity of Recombinant YY1. (A) Western blots of bacterial YY1 expression (top panel) and purification (bottom panel). Hours after addition of isopropyl-1-thio-β-D-galactopyranoside to log phase cultures are indicated at top. Purity of eluted protein was verified by silver staining after SDS-PAGE (right panel). LO, load on; FT, flow through and wash; EL, eluant. The position of molecular mass markers are indicated. (B) EMSA was performed with 100 nM YY1 and 10 mM consensus DNA probe. Requirement of zinc and magnesium for DNA binding of the recombinant protein is demonstrated in the upper panel and specificity of DNA binding in the lower panel. Recombinant protein was renatured in the presence of 0.1 mM EDTA prior to use in binding reactions, and reactions were then supplemented with zinc (0.2 mM) and/or magnesium (2 mM). To demonstrate sequence-specific DNA-binding, protein renatured in the presence of 0.2 mM zinc and 2 mM magnesium was used in binding reactions containing the following competitors: No competitor (NC), 1 pmol of double-stranded YY1 consensus (YY1C) or mutant (YY1M) DNA, 200 ng YY1 (αYY1) or PCNA (αPCNA) antibodies. (C) Varying concentrations of YY1 (indicated above panel) were combined in binding reactions with 10 nM consensus DNA (YY1C) or mutant consensus DNA (YY1M) probes and analyzed by EMSA.

Figure 5


A
C 0 1 2 3 4
C LO FT EL
kDa
75
50
37
B
Zn - + - +
Mg - - + +
YY1/DNA
P NC YY1C YY1M αYY1 αPCNA
YY1/DNA
C
[YY1]
0 1 nM 5 nM 10 nM 50 nM 0.1 μM 0.25 μM 0.5 μM 0.75 μM 1 μM 2 μM
YY1C
YY1M

3.2.2 Analysis of YY1 RNA-Binding Activity – In order to test the hypothesis that YY1 possesses RNA-binding activity, recombinant YY1 was combined with selected RNA probes in binding reactions and then analyzed by EMSA. Probes were chosen that possessed a uniform nucleotide composition (~ 50 % G/C content), were of appropriate size for use in EMSA analysis (< 200 nt), and were either relatively unstructured (β-Globin and GAPDH 3'-UTRs) or possessed significant secondary structure (5S rRNA). It should be noted that none of these probes were, or are necessarily suspected of being endogenous substrates of YY1 *in vivo*, but were chosen for initial analysis of YY1 RNA-binding activity because of their ready availability and the properties discussed above. Specifically, the probes used spanned the 3'-UTR of *Xenopus* GAPDH (136 nt) and β-Globin (130 nt), and the full length of the *Xenopus* 5S rRNA molecule (121 nt). The GAPDH and β-Globin probes are predicted to have little secondary structure, whereas the 5S rRNA contains significant secondary structure (Romaniuk, 1985). RNA binding reactions containing these probes were analyzed by RNA mobility shift assay. Analysis of binding reactions containing 0.5 μM recombinant YY1 and 10 nM radiolabelled GAPDH or β-Globin probe revealed that each formed a single prominent retarded complex that was specifically competed by addition of 1 pmol unlabelled YY1 consensus DNA (Figure 6). Under the same conditions, YY1 formed multiple complexes with the 5S rRNA which were competed by consensus DNA element (Figure 6). These data demonstrate that YY1 has the capacity to bind to disparate nucleotide sequences of both unstructured and highly structured RNAs.

To determine unequivocally the identity of the complexes described above, 0.5 μM recombinant YY1 was combined with 10 nM radiolabelled β-globin probe in the presence or absence of zinc and magnesium as well as with specific and non-specific competitors (Figure 7).

Complex formation depended on the presence of both zinc and magnesium ions. Furthermore, addition of YY1-consensus DNA competitor, or anti-YY1 antibody, reduced or abolished complex formation. Nonspecific competitors had no effect. These data are similar to what has been observed previously with the YY1-DNA interaction in that both magnesium and zinc are required for full binding activity, and also that addition of anti-YY1 antibody interferes with nucleic acid binding (Ficzycz et al., 2001).

Although the data presented above suggest that YY1 may interact with internal regions of RNA molecules, the possibility remains open that YY1 association with RNA could be affected by the presence or absence of the eukaryotic mRNA 5' 7-methylguanosine cap structure. The effects of 5'-cap modifications on YY1-RNA interactions were investigated using GAPDH and β-Globin RNA probes. Probes were prepared in the presence or absence of cap analogue, and the relative affinity for YY1 was analyzed in RNA-binding reactions containing a range of YY1 concentrations. RNA binding reactions were subjected to both EMSA and nitrocellulose filter binding assays (Figure 8 and Table 2). The analysis revealed no significant difference in binding affinity of YY1 to capped versus uncapped probes, either visible on the RNA-shift gel or in filter binding assays. Nitrocellulose filter binding assays yielded K_d values as follows: GAPDH uncapped, 14.9 ± 2.4 nM and capped, 17.4 ± 3.6 nM; β-globin uncapped, 47.8 ± 2.5 nM and capped, 49 ± 2.9 nM. Therefore, it is unlikely that interaction of YY1 with mRNAs *in vivo* involves direct recognition of the 7-methylguanosine cap structure. The affinity of YY1 for 5S rRNA was also determined (Figure 8 and Table 2) as 11.5 ± 2.9 nM. While apparent affinity of YY1 for 5S rRNA was higher than for either the GAPDH or β-globin probes, YY1 formed a number of distinct complexes with 5S rRNA, with a greater number of complexes appearing in reactions containing higher concentrations of YY1 (Figure 8).

Figure 6 - Analysis of YY1 RNA-Binding Activity. RNA-binding reactions containing 10 nM probe (indicated at bottom) in the presence (+) of 100 nM YY1 protein with addition (+) of 1 pmol double-stranded YY1 consensus DNA competitor (YY1E DNA) were analyzed by EMSA. Positions of YY1-RNA complexes and unbound free probe are indicated at left.

Figure 6

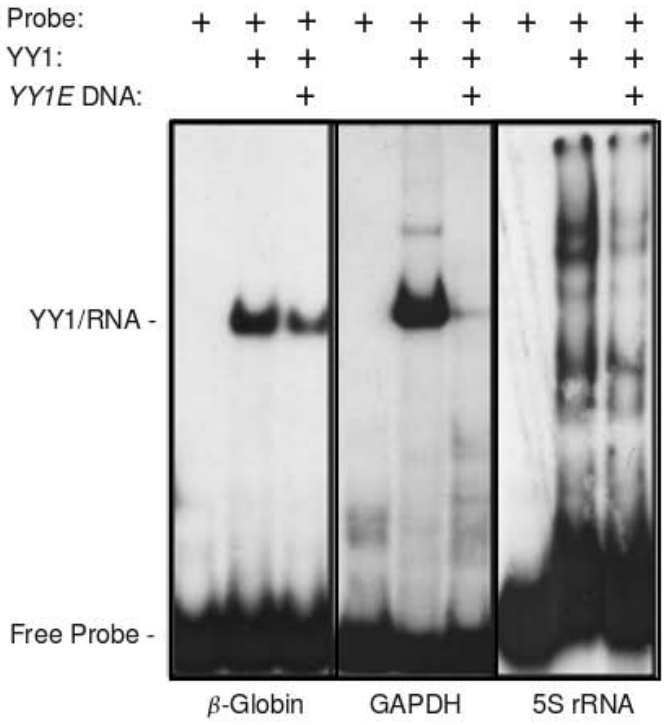


Figure 7 - Effect of Magnesium and Zinc Ions on RNA-Binding Activity of YY1. RNA-binding reactions containing 100 nM YY1 and 10 nM labelled β-Globin 3'UTR probe were subjected to EMSA. Binding reactions were carried out in the presence or absence of 0.2 mM zinc and/or 2 mM magnesium. Specificity of RNA-binding was analyzed by addition of competitors to binding reactions; no competitor (NC), 1 pmol of double-stranded YY1 consensus (YY1E) or mutant (YY1M) DNA, 200 ng of YY1 (αYY1) or PCNA (αPCNA) antibodies. The postition of the YY1-RNA complex and free probe are indicated at left.

Figure 7

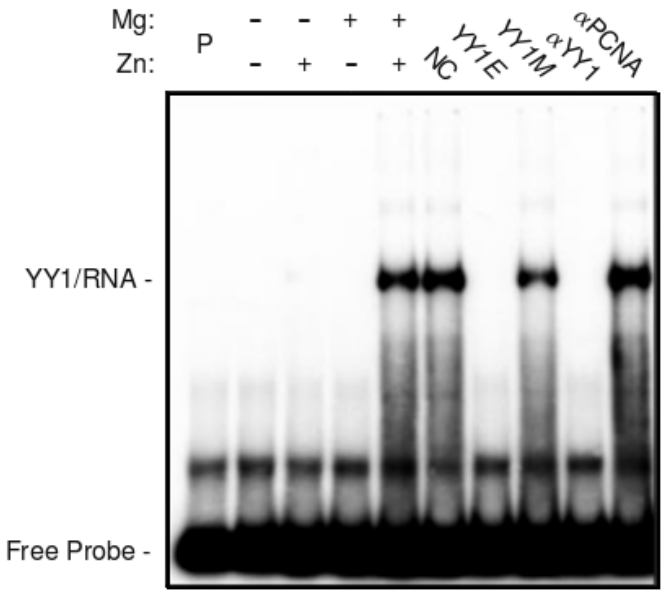
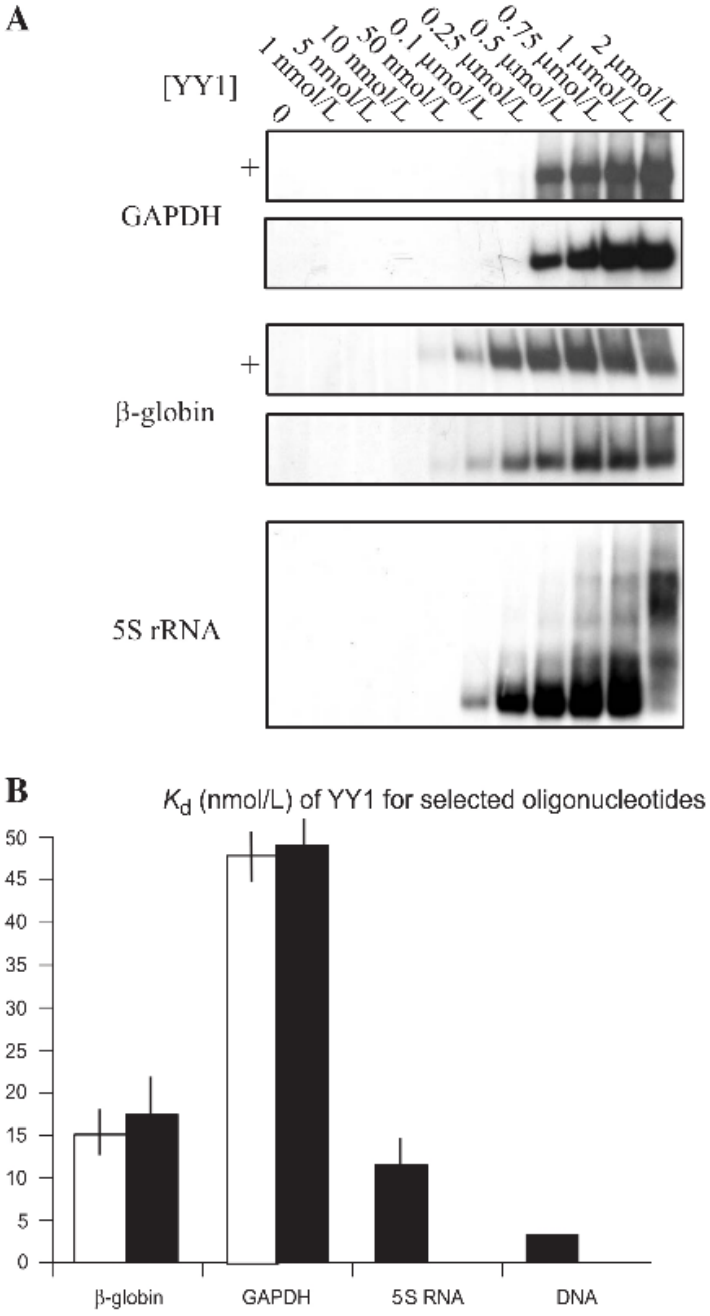


Figure 8 - Affinity of YY1 for Selected RNA Probes and Effect of 7-Methylguanosine Cap Structure on Substrate Binding Affinity of YY1. (A) RNA-binding reactions containing a range of YY1 concentrations (indicated at the top) and 10 mM RNA probe (indicated at left) were analyzed by EMSA. 5'-capped (+) or uncapped (-) probes are indicated at left. (B) Binding reactions were analyzed by filter binding assays and the K_d values are presented graphically. RNA or DNA probe substrate is indicated beneath the bar graph. For GAPDH and β-Globin, uncapped and capped probes are represented by white and black bars, respectively.

Figure 8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[YY1]
0
1 nmol/L
5 nmol/L
10 nmol/L
50 nmol/L
0.1 μmol/L
0.25 μmol/L
0.5 μmol/L
0.75 μmol/L
1 μmol/L
2 μmol/L
+
GAPDH
+
β-globin
5S rRNA
B
K_d (nmol/L) of YY1 for selected oligonucleotides
50
45
40
35
30
25
20
15
10
5
0
β-globin
GAPDH
5S RNA
DNA

3.2.3 YY1 Has High Affinity for U-rich RNA Substrates – The experiments described in section 3.2.2 demonstrate that YY1 has the ability to form intermolecular complexes with RNA; however, they shed little light on the substrate specificity of YY1. The following experiments analyze the affinity of YY1 for short (20 nucleotides) synthetic single-stranded and double-stranded oligoribonucleotide substrates with various sequences. One hypothesis was that YY1 may bind to RNA sequences complementary to the consensus DNA promoter element. To test this hypothesis binding reactions were assembled with various concentrations of YY1 and RNA probes containing the DNA consensus site (See Table 1) and analyzed by EMSA and filter binding assays. YY1 binding to single-stranded consensus as well as mutant RNA probes was barely detectable, and YY1 bound very weakly to both consensus and mutant duplex RNA probes (Figure 9 and Table 2).

The next experiments measured YY1 affinity for 20 nucleotide homopolymeric and heteropolymeric synthetic RNAs. Full descriptions of oligonucleotides used are provided in Table 1. EMSA and filter binding assays revealed high affinity of YY1 for U_{20} (K_d = 1.2 ± 0.2 nM), moderate affinity for A_{20} (K_d = 24.8 ± 1.3 nM), weak affinity for G_{20} (K_d = 114 ± 4 nM), and no detectable binding was observed with C_{20} (Figure 10 and Table 2). Analysis of binding to G_{20} by EMSA was not in accordance with filter binding data. It is surmised that gel shift assays were complicated by the presence of excessive secondary structure present in this probe, a postulate that is supported by the fact this probe was resistant to RNase T1 digestion (data not shown). It should also be noted in Figure 10 that in G_{20} containing binding reactions complex formation was not concentration-dependent with respect to YY1.

Next, YY1 affinity for the single-stranded dinucleotide combinations $(AC)_{10}$, $(AG)_{10}$, $(CU)_{10}$, and $(GU)_{10}$ were analyzed (Sequences in Table 1). The dinucleotide combinations $(AU)_{10}$ and

$(GC)_{10}$ form self-complementary duplexes and are analyzed below. YY1 bound to $(GU)_{10}$ with moderate affinity (K_d = 38.0 ± 1.5 nM), $(CU)_{10}$ and $(AG)_{10}$ with low affinity, and no binding to $(AC)_{10}$ was detected (Figure 10 and Table 2). These data reveal a preference for U-rich single-stranded probes.

The effect of RNA secondary structure on YY1 was examined in greater detail using duplex-forming RNA oligonucleotides. Six possible duplexes can be made from the homopolymeric and dinucleotide heteropolymeric RNAs described above (see Table 1). Each of these was prepared by labelling one of the two strands and annealing to the unlabelled complementary strand. In each case reciprocal experiments with the other strand labelled were performed to ensure the measured affinity was for the double-stranded probe. Additionally, double-strandedness was further verified by digestion with RNase A or T1 as appropriate (data not shown). YY1 bound with high affinity to the A:U duplex (K_d = 3.4 ± 0.1 nM), whereas binding to the C:G duplex was barely detectable by EMSA (Figure 10 and Table 2). No significant binding of YY1 to (AU):(AU), (GC):(GC), (AG):(CU), or (AC):(GU) RNA duplexes was observed in either EMSA or nitrocellulose filter binding assay (Table 2). Overall, the results shown in Figure 10 and the associated K_d values given in Table 2 demonstrate that YY1 exhibits direct, high affinity RNA-binding activity with some degree of sequence specificity toward U-rich substrates.

Figure 9 - Affinity of YY1 for RNAs Containing the YY1 DNA Consensus Sequence. RNA-binding reactions containing a gradient of YY1 concentrations (indicated at top) and 10 nM RNA probe (indicated at left) were subjected to EMSA. Probes used were: CF/R RNA, single stranded RNAs containing the YY1 DNA consensus sequence (F) or its reverse compliment (R); MF/R, single stranded RNAs containing the YY1 mutant DNA consensus sequence (F) or its reverse compliment (R); ds C RNA, double stranded RNA containing YY1 DNA consensus sequence; and ds M RNA, double stranded RNA containing YY1 mutant DNA consensus sequence. Full sequences and descriptions of probes used are given in Table 1.

Figure 9

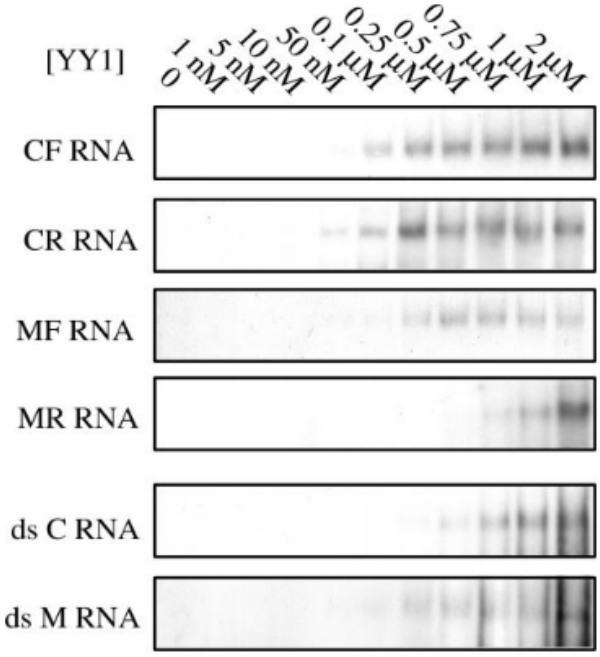

[YY1]
0
1 nM
5 nM
10 nM
50 nM
0.1 μM
0.25 μM
0.5 μM
0.75 μM
1 μM
2 μM
CF RNA
CR RNA
MF RNA
MR RNA
ds C RNA
ds M RNA

Figure 10 - Affinity of YY1 for Structurally Diverse Single Stranded and Double Stranded RNAs. RNA-binding reactions containing a gradient of YY1 concentrations (indicated at top) and 10 nM RNA probe (indicated at left) were subjected to EMSA. Probes used were: A/U/G/C(20), homopolymeric RNA 20-mer of the indicated nucleotide; AC/GA/CU/GU(10), hetropolymeric RNA 20-mer consisting of 10 repeats of the indicated dinucleotide; rA:rU, double stranded RNA consisting of U(20) hybridized to A(20); rC:rG, double stranded RNA consisting of C(20) hybridized to G(20). Full sequences and descriptions of probes used are given in Table 1. Only RNA substrates for which YY1 showed detectable binding by EMSA are shown.

Figure 10

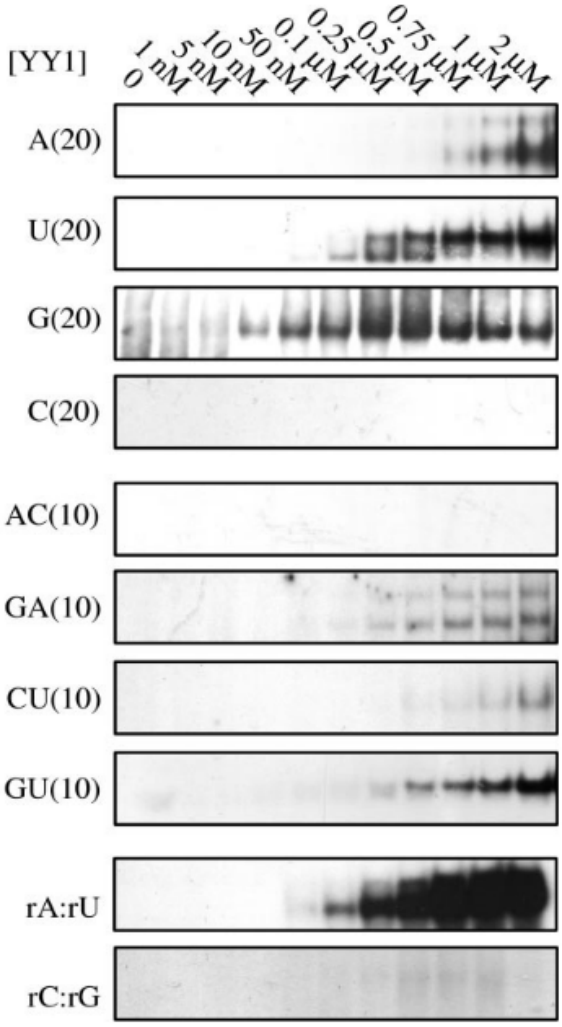

[YY1]
0
1 nM
5 nM
10 nM
50 nM
0.1 μM
0.25 μM
0.5 μM
0.75 μM
1 μM
2 μM
A(20)
U(20)
G(20)
C(20)
AC(10)
GA(10)
CU(10)
GU(10)
rA:rU
rC:rG

Table 2 – Dissociation Constants of YY1 for Various DNA and RNA Substrates

Oligonucleotide	**Dissociation constant (K_d) in nM**
Double-stranded consensus DNA element	3.2 ± 0.1
Double-stranded mutant consensus DNA element	54.9 ± 1.2
Double-stranded Heat Shock Response DNA element	> 150
CF RNA (consensus forward ssRNA)	> 150
CR RNA (consensus reverse ssRNA)	> 150
MF RNA (mutant forward ssRNA)	> 150
MR RNA (mutant reverse ssRNA)	> 150
ds C RNA (consensus dsRNA)	> 150
ds M RNA (mutant dsRNA)	> 150
A_{20} (poly-A ssRNA)	24.8 ± 1.3
U_{20} (poly-U ssRNA)	1.2 ± 0.2
G_{20} (poly-G ssRNA)	114 ± 4
C_{20} (poly-C ssRNA)	> 150
$(AC)_{10}$ (ssRNA)	> 150
$(AG)_{10}$ (ssRNA)	44.5 ± 1.7
(CU)10 (ssRNA)	73.4 ± 1.5
(GU)10 (ssRNA)	38.0 ± 1.5
A:U (dsRNA)	3.4 ± 0.1
C:G (dsRNA)	> 150
(AU):(AU) (dsRNA)	> 150
(GC):(GC) (dsRNA)	> 150
(AC):(GU) (dsRNA)	> 150
(CU):(AG) (dsRNA)	> 150

3.2.4 Stability of YY1-RNA Interactions – The affinity of YY1 for U-rich RNA substrates is described in section 3.2.3. To further characterize the RNA-binding activity of YY1, a radiolabelled U_{20} oligonucleotide probe (see Table 1) was used in binding reactions with recombinant YY1 under various conditions of temperature, ionic strength, and in the presence of detergents (Figure 11A). These experiments provide insight into the stability of YY1-RNA interactions as well as providing information to guide the design of further experiments. In RNA mobility shift assays, the relative amounts of detectable YY1-RNA complexes were highest when binding reactions were carried out at low temperature (0 – 4 °C). Only a slight decrease in complex stability was detected between 20 and 45 °C, and significant complex formation was still evident at 55 °C. These data demonstrate that YY1-RNA complexes are stable at temperatures within the physiological range of *Xenopus* oocytes, embryos, and adult tissues (12 – 25 °C). The persistence of RNA-binding activity at temperatures above this range is indicative of the thermodynamic stability of YY1-RNA complexes.

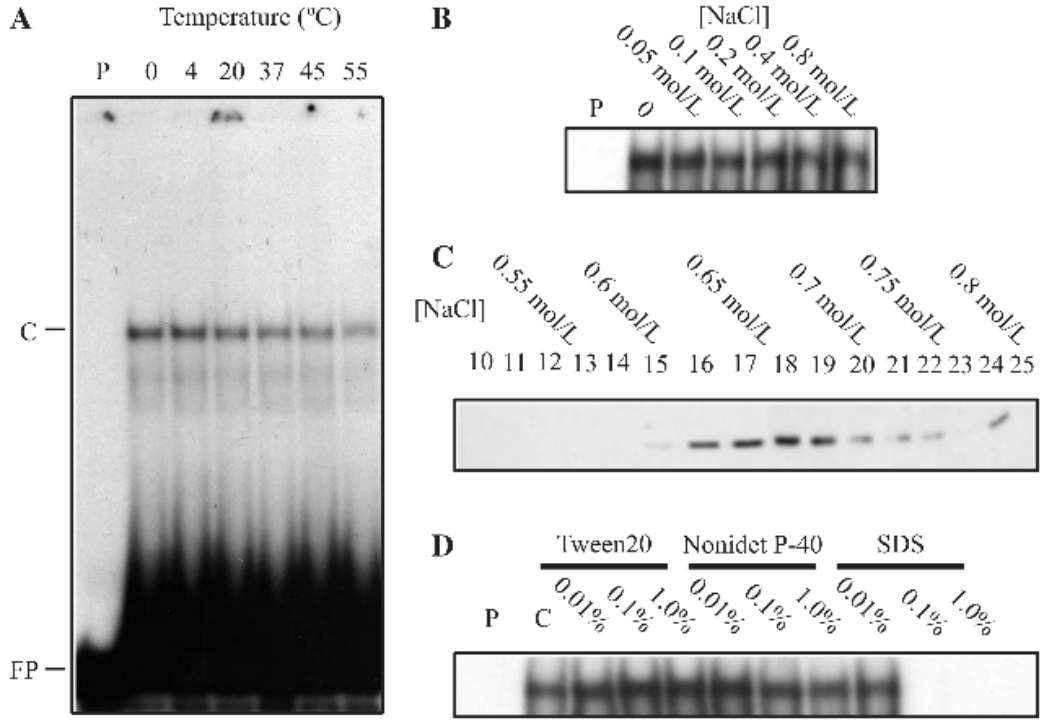
The ionic stability of YY1-RNA interactions was examined through a range of NaCl concentrations. YY1-RNA complexes appeared to be relatively stable up to 800 mM NaCl (Figure 11B). The ionic stability of YY1-RNA interactions *in vitro* were compared to the stability of YY1-RNA interactions in native mRNPs through the use of oligo-dT cellulose chromatography of oocyte lysates (Ficzycz and Ovsenek, 2002). In this assay, YY1-mRNPs are immobilized on oligo-dT cellulose matrix by virtue of the high-affinity interaction between the poly-T tracts of the matrix and the poly-A tails of mRNAs in YY1-containing mRNPs formed *in vivo* during oogenesis. Elution of YY1 from immobilized mRNPs using a NaCl gradient is an indirect measure of the stability of YY1-RNA interactions *in vivo*. YY1 disassociated from RNA at an NaCl concentration of approximately 600 mM to 800 mM (Figure 11C), similar to the

concentrations of NaCl that were shown to destabilize YY1-RNA interactions formed in *in vitro* binding reactions (Figure 11B). These data show that YY1-mRNA interactions formed in oocytes are extremely resistant to ionic perturbation, and suggest that once formed, YY1-mRNPs are highly stable complexes. Furthermore, these data support the hypothesis that the properties of recombinant YY1 analyzed by RNA-EMSA are representative of the properties of endogenous YY1 in complex with RNA in mRNPs *in vivo*.

The stability of the YY1-RNA interaction was further tested using detergents that destabilize hydrophobic intra- and inter-molecular protein interactions. YY1-RNA binding reactions were carried out in the presence of 0.01 % (w/v), 0.1 % (w/v) or 1 % (w/v) of Tween-20, Nonidet P-40, and SDS (Figure 11D). YY1-RNA complex formation was not disrupted by Tween-20, whereas a slight reduction in complex formation was observed with Nonidet P-40 above 0.1 %. Thus, the YY1-RNA interaction is stable in the presence of non-ionic detergents. SDS, a powerful ionic detergent, prevented complex formation at concentrations above 0.01 %, presumably via denaturation of YY1. Overall, the data presented in Figure 11 demonstrate that YY1-RNA interactions are highly stable under a wide range of harsh thermal and ionic conditions. These data were particularly useful in guiding the formulation of buffer compositions used in subsequent immunoprecipitation and analysis of YY1-mRNPs (see below).

Figure 11 - Thermal, Detergent, and Ionic Stability of YY1-RNA Interaction. RNA-binding reactions containing 100 nM YY1 and 10 mM poly-U(20) RNA probe were subjected to EMSA. (A) Binding reactions were carried out at the temperatures indicated above the panel. The positions of YY1-RNA complex and free probe are indicated. P, probe only; C, complex; FP, free probe. (B) Reactions were preformed with concentrations of sodium chloride as indicated above the panel. P, probe only. (C) Oocyte lysates were loaded onto oligo-dT cellulose and Western blotting analysis was performed on protein fractions eluted with a sodium chloride gradient. Fraction numbers and sodium chloride conentrations are indicated. (D) Reactions were preformed in the presence of various detergents as indicated. P, probe only.

Figure 11

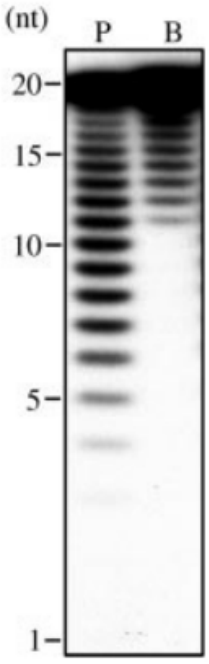

A
Temperature (°C)
P 0 4 20 37 45 55
C
FP
B
[NaCl]
0.05 mol/L
0.1 mol/L
0.2 mol/L
0.4 mol/L
0.8 mol/L
P 0
C
[NaCl]
0.55 mol/L
0.6 mol/L
0.65 mol/L
0.7 mol/L
0.75 mol/L
0.8 mol/L
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
D
Tween20
Nonidet P-40
SDS
0.01%
0.1%
1.0%
P C

3.2.5 The Minimal Binding Site of YY1 Contains Eleven Nucleotides –

To determine the minimal binding site of YY1, single-stranded U_{20} RNA was partially digested with RNase A, generating a population of probe fragments ranging in length from 1 to 20 nucleotides. Binding reactions were assembled with digested probe mixture and recombinant YY1 in the presence of nickel-charged NTA-agarose resin. YY1-RNA complexes formed in solution were recovered by binding of YY1 to the affinity matrix via the N-terminal His tag. The smallest fragment retained corresponds to the minimal binding site of YY1. Sequencing gel analysis of bound probe revealed YY1 bound fragments of 11 nucleotides and larger (Figure 12). Probe was not retained in control reactions without YY1 (data not shown). A minimum of 11 consecutive nucleotides are therefore required for binding of YY1 to RNA, and the YY1-RNA complexes observed with 20-mer probes (Figure 10) likely contain a single YY1 molecule.

Figure 12 - Determination of YY1 Minimal Binding Site. Poly-U(20) RNA probe (5 pmol) was partially digested with RNase A and added to RNA-binding reactions with 0.2 nmol YY1 in the presence of nickel-charged NTA-agarose, and sequencing gel analysis of bound poly-U RNA fragments was performed. Fragment sizes are indicated at left. nt, nucleotides; P, unbound probe; B, bound probe.

Figure 12

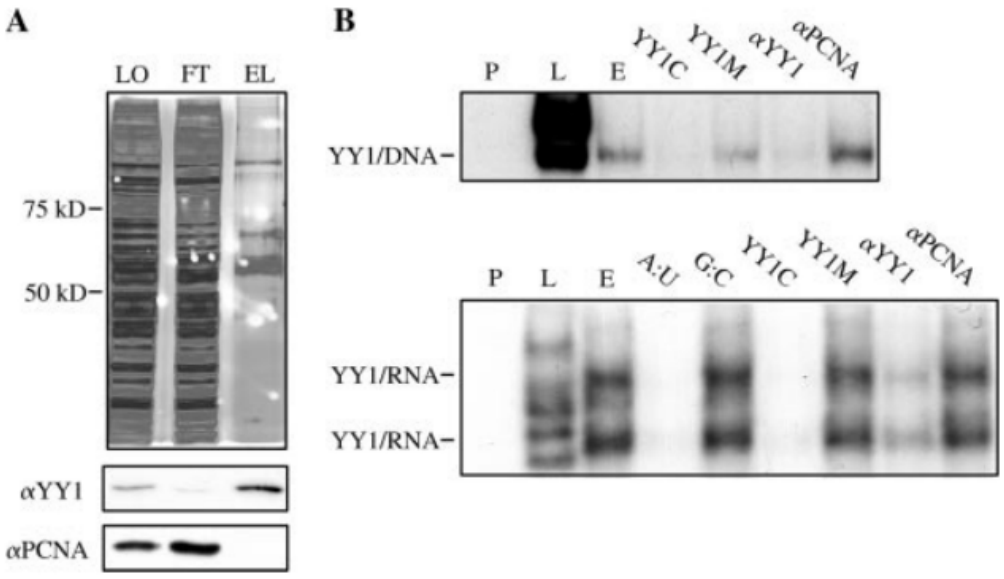


3.3 YY1 RNA-Binding Activity is Required for Assembly of YY1 into mRNPs –

Results obtained with recombinant protein described above were confirmed using native preparations of the endogenous protein isolated from oocyte mRNPs. Complexes isolated by oligo-dT cellulose chromatography (Ficzycz et al., 2001) were eluted and renatured, yielding an active preparation of isolated mRNP proteins, including YY1 (Figure 13A). Control analysis of one oocyte equivalent of this preparation by EMSA using YY1 DNA consensus probe revealed the presence of DNA binding activity (Figure 13B). YY1-DNA complexes were competed by specific oligonucleotide competitor or anti-YY1 antibody but were not affected by nonspecific competitors. Next, one oocyte equivalent of the mRNP preparation was analyzed for binding to U_{20} RNA probe. Two prominent retarded bands were observed that were competed specifically by A:U RNA duplex, YY1 consensus DNA, or anti-YY1 antibody (Figure 13B). Addition of G:C, for which recombinant YY1 displayed low affinity (Figure 10), did not compete for complex formation. Addition of YY1 mutant consensus DNA element or anti-PCNA antibodies also did not reduce complex formation. The results of nucleic acid binding experiments were consistent between both recombinant and native mRNP-associated YY1, with the exception that native YY1 formed two retarded bands. It is possible that other mRNP proteins, isolated by oligo-dT chromatography, are present in the retarded complexes. Together, these data clearly demonstrate that YY1 isolated from oocytes has the capacity to bind RNA directly and with high affinities similar to that for the DNA consensus.

Figure 13 - RNA-Binding Characteristics of Endogenous mRNP-Associated YY1. (A) mRNPs were obtained from stage VI oocytes by oligo-dT cellulose chromatography. Oocyte extract loaded onto oligo-dT matrix (LO), unbound material in flow-through (FT), and formamide eluant (EL) were analyzed by SDS-PAGE and silver staining (top panel) or Western blotting (lower panels). Position of molecular mass markers (top panel) and antibodies used (lower panels) are indicated at left. For both silver staining and western blotting, volumes were adjusted such that each lane contained one oocyte equivalent of material. (B) YY1-containing mRNP eluant was renatured and subjected to EMSA with 10 nM consensus DNA probe (upper panel). DNA-binding reactions contained one oocyte equivalent of material. Competitor DNAs (1 pmol) and antibodies (200 ng) added to binding reactions are indicated above the panel. The position of the YY1/DNA complex is indicated at left. RNA-EMSA (lower panel) was performed with renatured mRNP samples and 10 nM poly-U(20) RNA probe. The position of YY1/RNA complexes are indicated at left. The RNA (1 pmol), DNA (1 pmol), and antibody (200 ng) competitors added to reactions are indicated at the top of the panel. P, probe only; L, crude lysate; E, eluant.

Figure 13


A
LO
FT
EL
75 kD
50 kD
αYY1
αPCNA
B
P
L
E
YY1C
YY1M
αYY1
αPCNA
YY1/DNA
P
L
E
A:U
G:C
YY1C
YY1M
αYY1
αPCNA
YY1/RNA
YY1/RNA

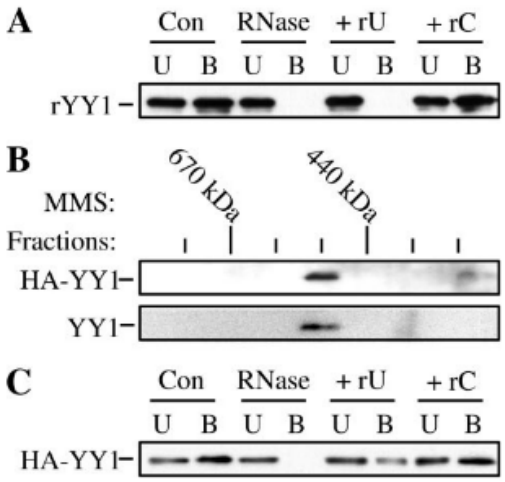
To demonstrate YY1 can associate directly with maternal mRNAs, *in vitro* binding reactions were assembled containing poly-A+ mRNA isolated directly from oocytes and recombinant YY1. Following incubation, reactions were subjected to oligo-dT cellulose chromatography to test for protein interaction with the maternal mRNA pool. Retention of YY1 on oligo-dT matrix demonstrated that YY1 was able to associate directly with maternal mRNA (Figure 14A). Treatment of reactions with RNase A abolished recovery of YY1 in bound fractions. The formation of YY1-mRNA complexes was effectively blocked by addition of U_{20} to binding reactions but not by C_{20}, results that are consistent with the direct RNA-binding experiments shown in Figure 10 and Table 2. Addition of U_{20} to reactions at the concentrations indicated did not significantly reduce total recovery of mRNA/mRNPs in bound fractions (data not shown). These *in vitro* data demonstrate that YY1 is able to bind directly to native maternal mRNA in the absence of other mRNP constituents.

The process of YY1-mRNP assembly was investigated *in vivo* by expression of HA-tagged YY1 from a plasmid construct (pHA3-YY1) microinjected into oocytes, and subsequent examination of cytoplasmic extracts by size exclusion chromatography (SEC) and oligo-dT cellulose chromatography (Figure 14B and 14C). The results show that nascent HA-YY1 assembled into poly-A+ mRNPs with a mean molecular mass of 480 kDa, identical in size to native YY1-containing mRNPs (Figure 14B) (Ficzycz et al., 2001). Additionally, HA-YY1 was retained on oligo-dT cellulose via interaction with maternally-derived endogenous poly-A+ mRNAs *in vivo* (Figure 14C). Cytoplasmic microinjection of RNase A into HA-YY1 expressing oocytes abolished retention of HA-YY1 on the oligo-dT matrix, demonstrating that isolation of HA-YY1 by oligo-dT cellulose is dependent on intact mRNA and is not due to anomalous interaction between oligo-dT and recombinant protein.

Time course experiments revealed HA-YY1 could be detected in mRNPs as early as 2 h after microinjection with peak levels of mRNP-associated HA-YY1 appearing after 12 h (data not shown). This indicates that the molecular machinery responsible for the assembly of mRNPs is completely functional in the mature oocyte and that epitope-tagged YY1 is competent to assemble into mRNPs. In some experiments, oocytes were enucleated after 8 h of expression, and nuclear and cytoplasmic extracts were analyzed by Western blot. HA-YY1 was found to be completely localized to the cytoplasm (data not shown), identical to what has been observed for native YY1 (Ficzycz et al., 2001; Ficzycz and Ovsenek, 2002). The assembly of HA-YY1 into mRNPs was significantly and specifically reduced by cytoplasmic microinjection of competitor RNA (U_{20}) (Figure 14C). Microinjection of RNAs for which YY1 displayed low affinity *in vitro* did not affect assembly of HA-YY1-mRNPs. The observation that high affinity RNA substrates are able to inhibit YY1-mRNP assembly *in vivo* clearly demonstrates that the RNA binding activity of YY1 is not only biologically relevant but is an absolute requirement for its assembly into mRNP complexes. These observations also show that protein-protein interactions between YY1 and other mRNP proteins are not sufficient for association with mRNPs, and that the most dominant process in YY1-mRNP association is direct interaction with maternal mRNA.

Figure 14 - Binding of YY1 to mRNA *in vitro* and *in vivo*. (A) *In vitro* binding reactions containing 10 pmol of recombinant YY1 and 0.5 μg of poly(A+) mRNA isolated from oocytes were subjected to oligo-dT cellulose chromatography. Bound (B) and unbound (U) fractions were analyzed by Western blotting for YY1. Some reactions were treated with 1 μg pf RNase A or 20 pmol of poly-U(20) or poly-C(20) competitors as indicated. Con, control. (B) Lysates from HA-YY1 expressing oocytes (upper panel) and control oocytes (lower panel) were subjected to size exclusion chromatography and fractions analyzed by western blotting. Anti-HA antibody was used in the upper panel and anti-YY1 antibody was used in the lower panel. Position of fractions and molecular mass standard (MMS) are indicated above the panel. (C) Lysates from HA-YY1 expressing oocytes were subjected to oligo-dT cellulose chromatography and bound and unbound samples analyzed by Western blotting with anti-HA antibody. HA-YY1 expressing oocytes were sham injected (Con) or microinjected with 20 ng RNase A (RNase), 5 pmol of poly-U(20) RNA (+rU), or poly-C(20) (+rC) RNA 4 h prior to preparation of extracts.

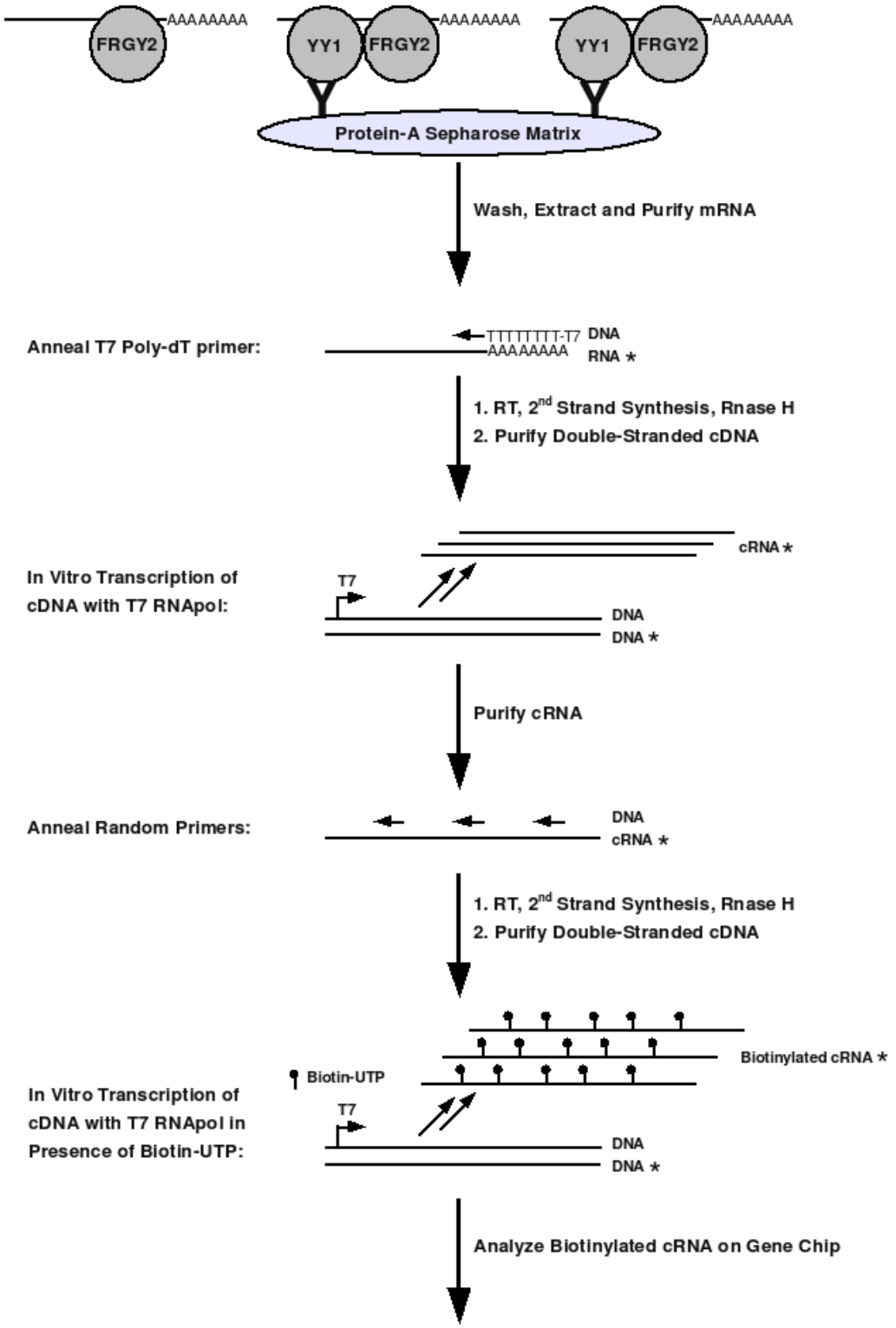
Figure 14


A
Con
RNase
+ rU
+ rC
U B U B U B U B
rYY1
B
MMS:
670 kDa
440 kDa
Fractions:
HA-YY1
YY1
C
Con
RNase
+ rU
+ rC
U B U B U B U B
HA-YY1

3.4 A Subset of Oocyte mRNAs Associate with YY1 in Cytoplasmic mRNPs - In order to determine the subset of oocyte mRNA bound by YY1, an RNA-immunoprecipitation/DNA microarray (RIP-CHIP) approach was used. In this approach, oocyte lysates are prepared under conditions that preserve the integrity of mRNPs. This lysate is then used in immunoprecipitation reactions with anti-YY1 antibody. This allows isolation of mRNP-YY1 along with the associated mRNAs. The isolated mRNA was amplified by two rounds of reverse transcription followed by *in vitro* transcription. The cRNA (copy RNA) was labelled by including biotin-UTP in the last *in vitro* transcription step. Total oocyte RNA was isolated and labelled in a single round of reverse transcription followed by *in vitro* transcription in the presence of labelled UTP. By hybridization of the labelled cRNA to a *Xenopus laevis* whole-genome DNA microarray it was possible to determine the subset of mRNAs present in both total oocyte and in YY1-IP samples. It is assumed that only mRNAs bound to YY1 and therefore isolated during anti-YY1 immunoprecipitation would be detected on the microarray hybridized with YY1-IP RNA. This assumption was then verified by Real-Time PCR of reverse transcribed mRNA isolated by anti-YY1 immunoprecipitation. The procedure for immunoprecipitation and labelling of YY1-associated mRNA is outlined in the flowchart shown in Figure 15.

Figure 15 – Flowchart of RIP-CHIP Analysis of YY1 Associated mRNA. The procedure used for RNA immunoprecipitation – DNA microarray (RIP-CHIP) analysis of YY1 associated mRNA is shown diagramatically. Polyadenylated transcripts associated with YY1 are isolated by immunoprecipitation, reverse transcribed using a T7-oligo-dT primer, and double-stranded cDNA is produced by treatment with *E. coli* DNA polymerase I. The resulting cDNA is transcribed *in vitro* using T7 polymerase and the resulting copy RNA (cRNA) is again reverse transcribed using random hexamers. The second-round cDNA is again transcribed *in vitro* using T7 polymerase in the presence of biotin-UTP generating biotinylated cRNA which is subsequently used in hybridization to the microarray. The procedure provides substantial amplification of mRNAs isolated by immunoprecipitation of YY1. Astrisks indicate RNA, cDNA, or cRNA strands with identical sequences.

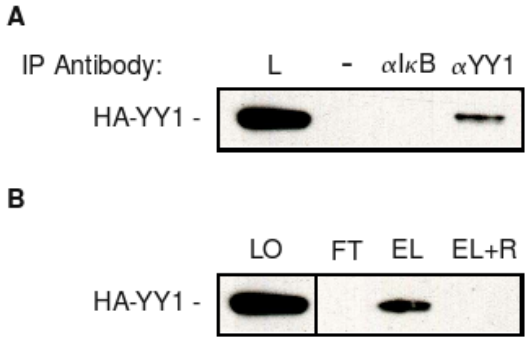
Figure 15


FRGY2
AAAAAAAA
YY1
FRGY2
AAAAAAAA
YY1
FRGY2
AAAAAAAA
Protein-A Sepharose Matrix
Wash, Extract and Purify mRNA
Anneal T7 Poly-dT primer:
TTTTTTTT-T7
AAAAAAAA
DNA
RNA *
1. RT, 2nd Strand Synthesis, Rnase H
2. Purify Double-Stranded cDNA
In Vitro Transcription of
cDNA with T7 RNApol:
cRNA *
T7
DNA
DNA *
Purify cRNA
Anneal Random Primers:
DNA
cRNA *
1. RT, 2nd Strand Synthesis, Rnase H
2. Purify Double-Stranded cDNA
Biotinylated cRNA *
Biotin-UTP
In Vitro Transcription of
cDNA with T7 RNApol in
Presence of Biotin-UTP:
T7
DNA
DNA *
Analyze Biotinylated cRNA on Gene Chip

3.4.1 Isolation of YY1-mRNPs by Immunoprecipitation – To demonstrate that YY1-mRNPs could be isolated by immunoprecipitation with anti-YY1 antibody, immunoprecipitations were performed using lysates from oocytes expressing HA-YY1. Detection of endogenous YY1 by Western blotting of anti-YY1 immunoprecipitates is complicated by detection of the immunoprecipitating antibody heavy chain in the same vicinity as YY1 protein. Therefore, since HA-YY1 has been shown to associate with mRNPs when expressed in oocytes (Section 3.3, Figure 14, and Figure 16) the utility of anti-YY1 antibody in isolation of YY1-mRNPs was examined by performing immunoprecipitations on lysates from HA-YY1 expressing oocytes. Figure 16 demonstrates that anti-YY1 antibody is capable of immunoprecipitating HA-YY1 from lysates (Figure 16A). Given that all expressed HA-YY1 was associated with mRNA as determined by oligo-dT cellulose chromatography (Figure 16B), these data support the claim that anti-YY1 antibody is effective for immunoprecipitation of mRNP-associated YY1.

<u>Figure 16</u> – Suitability of Anti-YY1 Antibody for Immunoprecipitation of YY1. To verify the ability of anti-YY1 antibody to immunoprecipitate YY1 from oocyte lysates, immunoprecipitations were performed on lysates from HA-YY1 expressing oocytes. Expression of HA-YY1 in oocytes was achieved by nuclear injection of 20 ng pHA3-YY1. After allowing 18 h for expression of tagged YY1, oocytes were lysed and the properties of HA-YY1 analyzed by immonoprecipitation with anti-YY1 antibody and oligo-dT cellulose chromatography. (A) HA-YY1 expressing oocyte lysates (L) were subjected to immunoprecipitation using anti-IκB (α κIB), anti-YY1 (αYY1), or with protein-A sepharose beads alone (-) and bound material was analyzed by Western blotting with anti-HA antibody. Position of HA-YY1 band in is indicated at left. (B) Lysates (LO) from HA-YY1 expressing oocytes were prepared in the presence of RNase inhibitor or supplimented with RNase A (+R) and applied to olgio-dT cellulose matrix. Unbound flow through (FT) and bound material eluted with formamide (EL) was analyzed by Western blotting with anti-HA antibody. Position of HA-YY1 band in is indicated at left.

Figure 16



3.4.2 RIP-CHIP analysis of YY1-mRNPs – Previously, the subset of oocyte mRNAs present in YY1-containing mRNPs was unknown. To address this issue, YY1 containing mRNPs and the mRNAs contained in them were isolated by immunoprecipitation with anti-YY1 antibody. The isolated mRNA was amplified, labelled, and used to probe a *Xenopus laevis* oligonucleotide microarray, an approach termed RIP-CHIP analysis. This approach allowed simultaneous identification of thousands of transcripts associated with YY1 in mRNPs. Bioinformatic and biochemical analysis of the functional roles of these transcripts is expected to provide considerable insight into the biological function of YY1 in mRNPs, and the functions of mRNPs in development. This analysis required comparison of the subset of transcripts present in YY1-mRNPs to the total set of transcripts expressed in the oocyte.

Total oocyte mRNA from five separate females and YY1 IP RNA from three separate females was analyzed using the *Xenopus laevis* Genome 2.0 oligonucleotide microarray (Affymetrix) and data from replicate samples was averaged. The datasets thus produced were used to generate the scatter plot shown in Figure 17. Messenger RNAs with similar levels in total oocyte RNA and YY1 IP RNA lie along a diagonal of slope = 1. Figure 17 shows a number of data points to the lower right of this line representing mRNAs with abundances higher in YY1 IP RNA relative to total RNA as well as a number of data points to the upper left of this line representing mRNAs with abundances lower in YY1 IP RNA relative to total RNA. The further toward the lower right a data point appears in the scatter plot the greater the likelihood the corresponding mRNA is present in YY1-containing mRNPs. Conversely, the further to the upper left a data point appears in the scatter plot the less likely it is that the corresponding mRNA is present in YY1-containing mRNPs. In the present work, only those data points that deviate substantially from the isometric diagonal were considered (the rationale for this approach is

discussed more fully in section 4.3). Messenger RNAs with a mean log(signal intensity) > 3 in total RNA and > 3.17 in YY1 IP RNA were considered likely to be present in YY1-mRNPs while mRNAs with mean log(signal intensity) > 3 in total RNA and < 2.7 in the YY1 IP RNA were considered unlikely to be present in YY1-mRNPs. Treatment of the data in this fashion resulted in identification of 1147 mRNAs present in YY1-mRNPs and 396 mRNAs not present in YY1-mRNPs. Of 1147 mRNAs identified as present in YY1-mRNPs, 730 (~63%) were unannotated transcripts while 417 (~37%) were known, characterized gene products. Among the 396 mRNAs identified as not present in YY1-mRNPs, 277 (~70%) were unannotated transcripts while 119 (~30%) were known, characterized gene products. The characterized transcripts identified in YY1-mRNPs are listed in Table 3 while characterized transcripts identified as not present in YY1-mRNPs are listed in Table 4.

In order to determine if transcripts present inYY1-mRNPs were associated with specific cellular functions, sequences were compared with the *Mus musculus* nucleotide database in order to retrieve Gene Ontology terms for transcripts with characterized protein products. Each transcript was categorized according to the retrieved Gene Ontology terms by molecular function (Table 5), cellular component (Table 6), and biological process (Table 7). Altogether, the functions associated with the protein products of YY1-bound transcripts were extremely diverse. Major molecular functions of YY1-associated transcript products (Table 5) were protein- and ATP-binding, reflecting the large number of regulatory protein transcripts (Table 3), and DNA-binding, reflecting the large number of transcripts encoding transcription factors. Also significant were transcripts encoding RNA-binding factors and zinc-binding proteins. Categorization of transcripts according to cellular component (Table 6) revealed the majority are nuclear proteins, with other major sites of residence being the cytoplasm, cell membrane, and

mitochondrion. Major biological processes associated with YY1-bound transcripts (Table 7) were transcription and regulation of transcription, again reflective of the large number of transcription factor transcripts; and also mitosis and cell division, indicative of a large number of transcripts for Cyclin proteins and other cell-cycle regulators (see Table 3). Altogether the results of Gene Ontology analysis shows the protein products of transcripts in YY1-mRNPs are associated with a very diverse set of cellular functions, however, major roles for these proteins are transcriptional regulation, cell cycle control, and signalling. These data are consistent with the hypothesis that maternally derived oocyte mRNAs present in YY1-mRNPs are utilized selectively during later development where their protein products direct important processes in growth and differentiation.

<u>Figure 17</u> - RIP-CHIP Analysis of Total mRNA and YY1 IP mRNA. A histogram was generated from mean signal intensity for each probe set (spot) on a *Xenopus* 2.0 Whole-Genome microarray probed with total oocyte RNA (Y-axis) plotted against mean signal intensity for each probe set in an identical array probed with anti-YY1 IP RNA. Control data comprises five independent replicates while anti-YY1 RNA data is derived from three independent replicates. Spots located toward the upper left of the diagram indicate mRNA which were not likely present in YY1-mRNPs while spots located further toward the bottom right indicate mRNAs which were highly enriched in anti-YY1 immunoprecipitates and are therefore likely present in YY1-mRNPs.

Figure 17

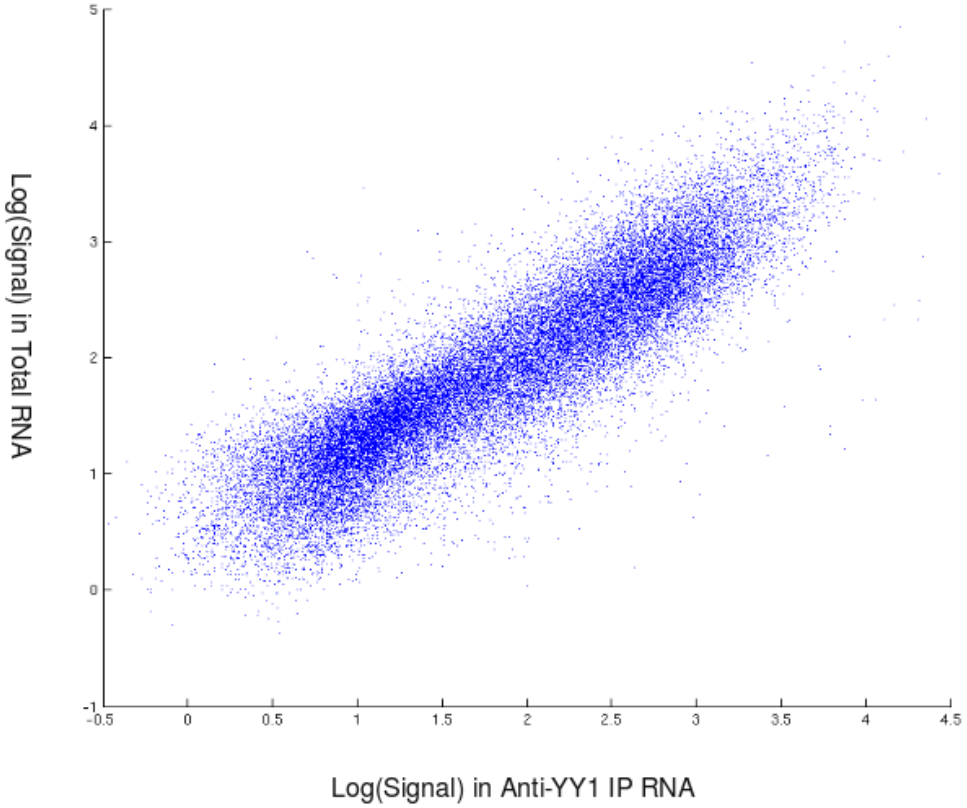

Log(Signal) in Total RNA
Log(Signal) in Anti-YY1 IP RNA
5
4
3
2
1
0
-1
-0.5
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

Table 3 – Oocyte mRNAs Present in YY1-Containing mRNPs

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
214231	16070.1	2091.03	71311.83	86804.84	xenopus laevis histone 4 mrna
93211090	7614.16	1420.42	51658.6	49856.94	Xenopus laevis linker histone H1A variant
214231	13658.98	4227.2	39383.77	48199.07	xenopus laevis histone 4 mrna
49115696	4881.78	379.67	26335.6	15541.37	Xenopus laevis survivin
46069704	8759.7	265.21	18902.3	11263.86	JC7 protein
28839017	9875.9	622.36	17683.5	5112.73	Xenopus laevis ribonucleotide reductase M2
49257964	9587.92	913.87	16317.2	3171.51	Xenopus laevis cyclin A1
65048	4145.72	216.92	15840.23	6458.4	Ribosomal protein L35a
51638543	2746.94	1558.07	15708.93	5129.17	Similar to cathepsin B
29483398	6094.42	270.84	15395.8	3093.5	Cyclin B1
77748264	1533.06	320.96	14225.07	6889.94	Xenopus laevis XNop56 protein
29482975	11296.48	1619.15	13812.87	5656.76	Similar to ubiquitin C
214240	3999.4	346.73	13631.67	3728.13	X.laevis ribonucleoprotein A1b protein
76779909	5909.26	638.38	13354.13	3597.01	Xenopus laevis arginine methyltransferase 1b
65056	1216.94	426.02	13272.13	5311.76	Xenopus laevis genes for 28S ribosomal RNAs
17479344	6392.96	732.65	13226.93	3179.45	Elogation factor 1 beta
214232	6178.82	654.77	13145.77	4134.25	X.laevis histone H1-like maternal (B4) protein
47717989	11626.96	1268.26	13139.07	2835.67	Xenopus laevis cyclin B4
51703883	1841.82	407.57	13104.17	4165.37	Xenopus laevis Wee1A kinase
38641396	1700.92	150.76	12961.33	11943.2	Xenopus laevis nuclear poly(A) binding protein 2 mRNA
31093161	3406.94	324.82	12927.13	2058.02	Citrate synthase
27371031	8285.18	422.36	12897.53	2135.74	Xenopus laevis similar to ribonucleotide reductase M2
33585635	2563.82	230.57	12734.57	3426.1	Xenopus laevis ubiquitin coniugating enzyme 3b
58701952	4303.1	275.54	12259.3	5289.54	Xenopus laevis transmembrane tight junction protein claudin
28436775	5595.38	1042.32	11749.4	5542.91	Xenopus laevis ribosomal protein L15
71681242	7378.64	755.48	11630.9	2951.12	Xenopus laevis Cyclin B2
13021625	3820.94	238.27	11630.6	3390.02	Xenopus laevis nonhistone chromosomal protein HMG-14
13021625	3819.68	125.2	11434.07	3491.17	Xenopus laevis nonhistone chromosomal protein HMG-14
70890462	3187.34	786.93	11246.23	2639.98	High-mobility group nucleosome binding domain 1
27371056	5810.84	433.56	11162.03	4631.59	Xenopus laevis small nuclear ribonucleoparticle-associated protein (snRNP)
29145230	3427.14	155.81	11086.77	6683.04	Ribosomal protein L35a
3219358	9459.74	456.57	11009.6	1796.75	Xenopus laevis geminin L

Table 3 – Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
72370886	3817.2	146.91	6568.43	1173.53	Cytokeratin type II
1685279	7858.48	726.63	6552.13	1241.24	Xenopus laevis histone H2A.z variant
24090401	2819.14	473.5	6426.57	2223.9	Similar to high mobility group box 2
28302172	4170.04	188.9	6365.7	2593.19	Xenopus laevis splicing factor, argininеserine-rich 3
24083245	6608.44	496.99	6289.57	3525.57	EIF4G-related protein NAT1
12473585	6186.2	1269.93	6259.6	2287.23	Splicing factor, argininеserine-rich 2
27371290	6630.68	426.31	6257.43	1195.77	Xenopus laevis suppressor of initiator codon mutations, related sequence 1
2370592	7679.76	1612.63	6252.3	2340.56	Translation initiation factor eIF4A II
23867779	6006.34	578.46	6232.77	1634.1	Xenopus laevis Claudin4L2
49256097	1614.48	149.68	6231.67	1951.15	Xenopus laevis similar to ribonucleotide reductase M1
28277249	1583.2	506.59	6171.27	1850.19	Xenopus laevis similar to myo-inositol 1-phosphate synthase A1
47122871	3039.32	503.87	6138.9	1546.59	Xenopus laevis Cdc6-related protein
64679	3974.4	536.04	6038.13	1256.94	Enolase 1, alpha
27506837	16544.28	2790.58	5996.53	2203.63	Ornithine decarboxylase 1
15273577	6207.8	543.51	5934.4	2660.5	Ribosomal protein S27
46329522	3426.52	248.19	5826.23	1748.41	Xenopus laevis maternal B9.10 protein
11175346	6268.68	1238.83	5792.67	1056.43	Insulin-like growth factor-1 receptor beta subunit
27924421	9673.88	508.57	5729.27	745.36	Xenopus laevis similar to ubiquitin C
31090084	1997.68	193.31	5728.27	1349.7	Elongation factor 1 gamma
42810157	1793.74	505.92	5699.4	1711.42	Cu-Zn superoxide dismutase
28277296	2358.68	316.75	5646.67	984.71	Xenopus laevis similar to RNA cyclase homolog
1125740	2270.38	295.02	5598.43	703.19	Xenopus laevis fibroblast growth factor receptor ligand 1 (FRL1)
70893697	5923.2	370.48	5565.97	1313.82	Ribosomal protein S8
12004633	1897.96	222.24	5514.13	1136.27	Xenopus laevis reptin mRNA
27371210	6256.52	616.47	5483.63	642.22	Xenopus laevis guanine nucleotide binding protein, beta 2
50416239	9786.92	725.07	5482.27	1404.86	Xenopus laevis uroplakin 1B
46086487	1079.26	73.26	5463.67	2152.95	Targeting protein for Xklp2
7030709	3033.7	224.58	5461.33	1390.54	SnRNP C protein
70672124	6097.6	189.66	5451.93	1032.52	Xenopus laevis comp24 mRNA
27371151	3232.7	978.13	5448.57	2203.76	Xenopus laevis ribosomal protein L19
27503840	6859.38	330.34	5401.37	940.41	Xenopus laevis nuclease sensitive element binding protein 1
15273577	4606.92	389.08	5391.57	2568.09	Ribosomal protein S27
46377817	2295.86	344.97	5363.8	2117.26	Elongation factor-1 delta
32450383	2977.72	91.27	5348.53	2050.8	Xenopus laevis high-mobility group box 1

Table 3 – Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
56269922	1525.64	207.78	10878.33	3179.68	Xenopus laevis NM23 nucleoside diphosphate kinase
15553376	7469.18	952.27	10631.73	2821.58	Xenopus laevis claudin A (cldna)
70894583	4049.82	556.17	10542.4	2358.27	Similar to ribonucleotide reductase M1
21898547	4950.96	440.67	10476.73	5412.35	Xenopus laevis survivin
6007765	1628.42	171.4	10411.13	2453.01	Xenopus laevis casein kinase I epsilon (CKIe)
52139069	2724.5	198.86	9894.83	1521.61	Xenopus laevis tyrosine phosphatase Cdc25A
95017016	5263.84	663.95	9845.43	851.06	XLCL2
51950031	6413.32	424.41	9584.1	1521.42	Xenopus laevis geminin H
27371294	1785.74	319.87	9243.03	2340.16	Xenopus laevis phosphoribosylaminoimidazole carboxylase
3721939	3264.4	64.28	9123	2081.46	Xenopus laevis NO27
29482975	10235.9	803.45	9095.7	2602.39	Similar to ubiquitin C
51607232	3021.26	207.4	9083.4	3011.14	H3 histone, family 3A
28436919	3432.34	243.06	8984.6	2396.38	Xenopus laevis ATPase, H+ transporting, lysosomal, beta isoform 2
213955	1588.2	116.71	8957.57	1652.16	Xenopus laevis Na+K+-transporting ATPase beta subunit
32450492	5251.48	491.84	8773.33	2833.59	Xenopus laevis ribosomal protein S11
10755414	1356.04	167.53	8725.07	3556.07	Similar to heterogeneous nuclear ribonucleoprotein U
70893769	7162.5	1279.03	8716.37	4993.78	Translation initiation factor eIF4A II
64918	4336.04	542.83	8521.7	3322.69	X.laevis nucleolar-localized protein NO38
46086199	4911.06	361.27	8416.57	1336.6	Ribosomal protein S19
52354794	4483.34	159.48	8312.27	1941.37	Xenopus laevis thyroid receptor interacting protein 7
3650201	2825.42	317.79	8202.97	1944.1	Xenopus laevis Zic2 protein
108752876	10186.66	732.77	8154.8	1388.52	Similar to ubiquitin C
28302172	3820.38	153.14	8011.67	3783.29	Xenopus laevis splicing factor, arginineserine-rich 3
33417131	1976.52	175.62	7768.57	2353.72	Xenopus laevis string of pearls
1136598	3884.76	735.47	7661.8	3084.22	Xenopus laevis glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
68163352	2623.18	201.8	7629.9	3418.44	Xenopus laevis xSurvivin1B
8319436	4447.46	187.71	7591.23	416.34	H3 histone, family 3B
13567571	2235.14	148.12	7501.1	1256.51	FXR1
4580025	3555.04	486.33	7296.63	2212.13	Xenopus laevis CCCH zinc finger protein C3H-4
54648593	3535.4	275.2	7242.17	2088.21	Xenopus laevis alpha-tubulin
28436777	6058.64	674.55	7224.2	1714.78	Xenopus laevis ribosomal protein L9
70672131	3363.58	178.28	7038.53	1384.29	Xenopus laevis soco10
25991259	2133.42	98.17	6990.83	2303.38	Xenopus laevis Erp1
7248364	6070.08	539.94	6636.63	742.01	Xenopus laevis proliferating cell nuclear antigen subtype1

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
33646871	8588.52	759.49	5300.9	805.75	Solute carrier family 25, member 5
49493477	1001.76	72	5253.47	3499.75	Transcription factor XTCF-3b
4191595	2152.26	288.98	5207.83	1224.01	Xenopus laevis chromosome condensation protein XCAP-G
54632056	6352.24	736.41	5197.47	2326.79	Claudin 6
46077446	11081.92	924.72	5136.7	817.71	CCAAT box transcription factor p122 subunit
28280048	3612.12	362.41	5070.7	1936.44	Xenopus laevis nuclear RNA helicase
28302265	6484.48	467.09	5064.6	1283.02	Xenopus laevis chromobox homolog 3
108752597	3716.92	1292.92	5050.6	558.52	Phosphoglycerate kinase 2
68163354	2728.3	252.19	5046.07	1487.06	Xenopus laevis xSurvivin2B
49116087	5777.64	337.9	5044.9	1521.73	Xenopus laevis Xenopus RPA interacting protein alpha
49114897	3793.72	545.01	5018.87	444.99	Xenopus laevis nucleoplasmin
50603911	2139.3	132.62	5009.43	951.77	Xenopus laevis DEAD (Asp-Glu-Ala-Asp) box polypeptide 55
88890179	3563.98	206.62	5006.3	1883.47	Methionine adenosyltransferase II
49257201	1961.9	44.77	4998.57	1290.1	Xenopus laevis C protein
46069822	5010.04	383.52	4950.1	1400.2	Ribosomal protein S12
28175725	2489.1	148.59	4936.23	1623.82	Xenopus laevis cyclin-dependent kinase 9 (CDC2-related kinase)
6942204	11997.42	893.3	4921.03	2346.69	Xenopus laevis securin
43387384	5246.64	351.37	4900.73	981.42	Tumor protein, translationally-controlled 1 (tpt1)
213955	3074.78	134.74	4829	1325.44	Xenopus laevis Na+K+-transporting ATPase beta subunit
58702035	6399.92	271.37	4701.13	1592.41	Xenopus laevis D7 protein
37508460	2283.78	253.94	4656	1282.19	Tumor protein, translationally-controlled 1 (tpt1)
27924190	1387.46	147.41	4628.73	1231.96	Xenopus laevis proteasome (prosome, macropain) subunit, alpha type, 4
27924188	6525.66	327.19	4602.77	795.55	Xenopus laevis sans fille
49116013	3218.82	108.13	4540.13	1172.65	Xenopus laevis Cdc45
3764086	2219.64	89.74	4505.5	1655.73	Xenopus laevis 13S condensin XCAP-D2 subunit
95010831	3129.4	300.31	4478.2	1305.27	Ribosomal protein S9
27515988	4412.14	330.66	4452.23	507.05	Polo-like kinase
31451468	4317.62	553.43	4431.37	177.71	Creatine kinase, brain
32450617	1193.42	80.63	4395.87	960.06	Xenopus laevis von Hippel-Lindau binding protein 1
27694791	1707.1	164.28	4372.6	1240.46	Xenopus laevis mitochondrial Ca2+-dependent solute carrier
27735387	3407.56	361.14	4362.47	882.2	Xenopus laevis ribosomal protein S6
46080137	1091.16	218.55	4308.17	1860.19	Nucleolin
54037958	2461.42	176.15	4293.73	466.68	Xenopus laevis enhancer of zeste
95019766	2486.52	88.72	4250.47	902.27	CCR4-NOT transcription complex, subunit 8

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
46087062	2425.34	104.59	4213.13	682.28	Annexin 4
47937641	1881.48	277.41	4205.77	1533.2	Xenopus laevis SOX3 protein
47682266	3088.42	194.49	4165.97	505.38	Xenopus laevis integrin beta-1 subunit
28436791	6449	524.47	4140.4	1589.36	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit
46045094	3677.62	205.02	4137.27	696.38	Eukaryotic translation initiation factor 3 subunit 10
28461385	3143.96	542.38	4127.93	105.87	Xenopus laevis similar to tubulin, beta, 2
6479975	1528.02	118.15	4070.3	2466.98	High density lipoprotein binding protein (vigilin)
49114986	1953.34	218.11	4052.63	942.05	Xenopus laevis fibronectin protein
46067384	1394.56	117.41	4037.53	2287.81	Ribosomal protein S21
32450031	3163.22	132.5	4023.4	1596.76	Xenopus laevis ribosomal protein L30
32484262	3103.72	248.83	4020.13	649.67	Xenopus laevis elongation factor 1 gamma
214240	2253.42	175.15	4004.73	1448.66	Ribonucleoprotein A1a
2852393	1004.4	36.37	3942.2	1101.77	Xenopus laevis retinoid X receptor-interacting coactivator xSRC-3
24087822	1215.66	72.47	3919.97	659.26	RNA binding motif protein 7
37497669	2524.86	152.68	3881.57	1713.58	SnRNP C protein
6006269	2905.54	182.52	3873.57	832.7	Xenopus laevis TAF-Ibeta2
32450164	2997.04	268.44	3863.23	740.78	Xenopus laevis vaccinia related kinase 1
2408144	1935.26	113.21	3862.4	463.91	Xenopus laevis enhancer split groucho (ESG)
47125180	1231.88	151.14	3857.43	1212.89	Xenopus laevis MYC II
49670411	3548.24	322.57	3839.87	2443.97	Xenopus laevis ribosomal protein L13
29479554	3332.26	552.07	3808.27	523.14	Uncoupling protein 2
8572737	1196.38	165.14	3807.33	1478.52	Xenopus laevis twisted gastrulation
21074557	5898.26	502.33	3788.8	1205.09	Nucleosome assembly protein 1 p56A
27696874	2745.48	338.59	3769.87	404.41	Xenopus laevis karyopherin alpha 2
37293019	2096.32	40.98	3756.33	999.23	Nuclear membrane protein XMAN1
46067384	3066.42	263.82	3741.6	1580.72	Ribosomal protein S21
27924332	1635.3	245.16	3682.43	1573.78	Xenopus laevis chaperonin containing TCP1, subunit 5 (epsilon)
8886478	1925.14	71.48	3669.93	808.46	Xenopus laevis Int-6 protein
6652990	1272.5	132.35	3667.13	674.41	Xenopus laevis axin-related protein
46086725	3274.38	175.41	3632.43	1179.3	Ribonucleoprotein A1a
37279038	5669.24	260.81	3623.3	2006.15	Enhancer of rudimentary homologue ERH
27515988	5210.6	245.13	3606.4	630.72	Polo-like kinase
29481578	7507.18	362.47	3594.03	1398.39	Tumor-associated calcium signal transducer 1
95017209	2332.92	190.88	3578.37	1670.51	Ribosomal protein L12

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
46052482	5441.74	385.67	3565.47	879.66	eterogeneous nuclear ribonucleoprotein AB
76780391	2649.98	133.41	3552.17	669.82	Xenopus laevis mitochondrial malate dehydrogenase 2a
27503240	1320.5	53.6	3545.13	868.12	Xenopus laevis guanine nucleotide binding protein, alpha 11
27503417	6835.6	924.7	3534.33	1518.85	Xenopus laevis creatine kinase, brain
46067034	1379.88	184.44	3511.97	1981.56	CCAAT box transcription factor p122 subunit
28175909	1037.94	115.39	3483.1	714.52	Cdc6-related protein
6939743	2982.78	547.14	3478.13	599.38	Xenopus laevis mRNA for gp37
28302335	1042.5	82.82	3473.53	975.81	Xenopus laevis solute carrier family 30 (zinc transporter), member 1
49491202	1454.4	114.99	3463.93	1254.59	Mitotic phosphoprotein 90
27370991	2191.42	565.87	3463.07	857.1	Xenopus laevis tyrosine 3-/tryptophan 5-monooxygenase activator zeta
47123095	1545.76	208.25	3461.27	779.3	Xenopus laevis DBF4 protein
9988417	3368.06	229.34	3438.97	278.19	Xenopus laevis POLE2 mRNA for DNA polymerase epsilon subunit B
21073059	1569.08	175.69	3430.07	922.61	Forkhead activin signal transducer 1
2231292	4432.02	410.04	3403.33	340.8	Xenopus CDC47-2p
31451468	5311.94	963.2	3392.73	123.18	Creatine kinase, brain
32450399	4018	591.27	3371.83	241.32	Xenopus laevis lactate dehydrogenase 2
50414598	4229.36	217.26	3371.03	440.15	Xenopus laevis glycogenin 1
51609100	2507	295.07	3369	1564.21	S-Adenosylmethionine decarboxylase 1A
28302265	2636.26	270.14	3366.33	1286.86	Xenopus laevis chromobox homolog 3
72375437	1652.82	284.1	3340.57	1205.3	Ribosomal protein L7a
88890179	2365.34	250.21	3321.2	1254.15	Methionine adenosyltransferase II
17500384	2075.14	115.27	3311.67	823.32	SMC-like 1
32822848	2376.06	497.15	3307.63	1824.25	Xenopus laevis ribosomal protein L4-b
27371202	2582.72	440.11	3298.63	966.59	Xenopus laevis ubiquinol-cytochrome c reductase
54311174	4549.56	447.95	3278.13	481.87	Xenopus laevis translation initiation factor SUI1
46250229	2645.56	472.16	3275.03	1056.99	Xenopus laevis XB-cadherin
9726371	6417.9	557.07	3211.6	225.23	Variant histone H2A.Zl1
4324618	3084.72	387.65	3209.27	201.59	Xenopus laevis (SLBP2)
27503216	5006.74	494.01	3161	1435.44	Xenopus laevis similar to ribosomal protein L5
50417441	1465.48	116.21	3160.5	2311.96	Xenopus laevis X1-c-ets-2b protein
26043094	1257.28	135.17	3155.93	603.04	Cbl proto-oncogene protein
46381136	3266.88	125.6	3142.53	401.4	Ubiquitin-conjugating enzyme E2H
52354799	2844.02	215.97	3102.4	75.42	Xenopus laevis kinesine
24093178	1626.28	240.62	3097.83	582.06	Apoptosis inhibitor 5

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
27371202	5371.32	162.08	3087.6	491.54	Xenopus laevis ubiquinol-cytochrome c reductase
33585856	1424.08	148.62	3077.23	2112.49	Xenopus laevis ribosomal protein S10
1469525	1318.6	71.9	3060	729.53	Xenopus laevis XMCM7
28302200	4115.06	299.98	3055.33	279.07	Xenopus laevis NHP2 non-histone chromosome protein 2-like 1
51895486	2071.38	237.74	3052.4	592.49	Xenopus laevis merlin mRNA
57032510	4713.16	526.33	3030.87	176.5	Xenopus laevis variant histone H2A.Zl1
47937627	2715.98	221.65	3023.33	182.26	Xenopus laevis similar to HIV-1 rev binding protein 2
39738303	1077.96	123.5	3022.3	799.15	BRM protein
1731731	3686.4	289.01	3019.53	560.83	Xenopus laevis DNA (cytosine-5-)-methyltransferase
31090578	2176.02	387	3011.77	382.07	Aldolase C, fructose-bisphosphate
50603696	1028.3	127.57	3008.07	600.25	Xenopus laevis protein kinase C substrate 80K-H
46250184	1158.8	135.95	3004.33	735.42	Xenopus laevis ras protein
50417439	3013.68	195.78	3003.6	551.05	Xenopus laevis cyclin A2
72373081	3972.96	398.5	2998.4	284.42	Ran binding protein 1
28175313	1179.64	108.62	2993.77	609.19	Xenopus laevis similar to CDC42 effector protein (Rho GTPase binding) 2
58701930	1835.52	273.65	2981.23	94.32	Xenopus laevis cyclin E3
51607260	4949.82	921	2973.17	504.87	Proliferating cell nuclear antigen
27370896	2428.9	429.09	2972.6	690.9	Xenopus laevis polyadenylate-binding protein-interacting protein 2
46044212	1858.88	214.64	2967.7	1283.21	Heterogeneous ribonuclear particle protein
46376752	2295.9	185.2	2955.53	512.82	Kinesin family member 2C
29835711	2098.42	197.31	2947.23	669.35	Peptidylprolyl isomerase B
12748332	6615.86	384	2935.47	881.5	Cyclin B5
28422220	1719.8	84.01	2929.23	2038.86	Xenopus laevis similar to signal sequence receptor, beta
4584794	1856.84	129.48	2909.43	364.88	Xenopus laevis XL-INCENP
46249497	1812.08	143.79	2904.73	1666.82	Xenopus laevis coronin homolog
27694839	3137.26	224.32	2859.9	621.4	Xenopus laevis pyruvate kinase, muscle
49495535	1617.12	122.98	2858.83	60.72	Xenopus laevis, Similar to kinesin family member 23
28422353	1723.94	33.64	2834.7	440.2	Xenopus laevis N-acetyltransferase ARD1 homolog
76779560	1521.28	267.59	2829.1	574.43	Xenopus laevis amadillo repeat-containing protein
95020132	2626.08	330.85	2826.93	1511.8	Ribosomal protein L13a
26985224	1809.66	298.86	2825.87	411.9	Xenopus laevis flotillin 1a (flot1a)
72360914	2362.08	234.79	2807.53	808.62	Ribosomal protein L24
57032510	6296.84	539.66	2797.93	185.01	Xenopus laevis variant histone H2A.Zl1
27503905	2841.02	252.64	2779.27	1168.43	Xenopus laevis protein associated with PRK1

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
58702023	3275.96	121.32	2774.07	599.64	Xenopus laevis TBP-like factor
31090318	2699.56	276.39	2766.93	1162.06	ATP dependent RNA helicase
27503899	4932.08	314.49	2757.13	976.83	Xenopus laevis similar to cell division cycle 20 homolog
28277234	1061.08	49.56	2752.83	810.01	Xenopus laevis ubiquitin-conjugating enzyme E2I
27370999	2606.98	188.87	2738.33	650.42	Xenopus laevis similar to enolase 1, alpha non-neuron
49670416	1643.02	153.91	2706.83	428.85	Xenopus laevis CNDP dipeptidase 2 (metallopeptidase M20 family)
27371264	8363.7	940.26	2672.73	309.81	ATP synthase, mitochondrial F0 complex, subunit c (subunit 9) isoform 3
50603677	1057.08	48.2	2670.87	201.98	Xenopus laevis catenin, beta like 1
56550341	1663.06	203.71	2670.57	1062.64	Xenopus laevis fox factor (FoxI2 gene)
63021616	4856.88	331.52	2657.23	325.86	Xenopus laevis cystatin mRNA
11034806	1172.9	150.87	2656.43	1316.26	Xenopus laevis RNA-binding protein Y14
70895127	4409.32	486.46	2588.37	318.1	Heterogeneous nuclear ribonucleoprotein AB
27882616	5945.5	452.23	2583.07	527.05	Xenopus laevis solute carrier family 25, member 5
27506837	9043.6	1391.74	2582.9	1208.78	Ornithine decarboxylase 1
27694781	3575.76	325.94	2581.17	254.17	Xenopus laevis ribosomal protein L8
28386022	3852.54	189.31	2573.3	278.1	Xenopus laevis karyopherin alpha 2
27370910	1758.52	275.68	2567.07	209.68	Xenopus laevis Adenylate kinase 2
31090541	6097.38	341.35	2554.37	1042.39	Stathmin 1oncoprotein 18
32395650	1202.92	134.22	2553.27	608.23	Xenopus laevis negatively regulating zinc finger protein (NZFP)
46249650	1245.3	271.23	2509.57	209.68	Xenopus laevis RING finger protein
27368040	1355.54	45.01	2475.3	358.79	Xenopus laevis kinetochore spindle checkpoint protein Nuf2
17373244	1771.86	71.01	2470.67	637.93	U5 snRNP-specific 40 kDa protein
46032198	5689.9	366.79	2470.47	950.42	Ubiquitin-conjugating enzyme E2G
27769400	1625.82	303.68	2457.93	566.12	Xenopus laevis ribosomal protein S1a
108752807	1979.68	198.67	2451.83	914.98	MARCKS-like protein
27371246	4796.24	1116.64	2431.47	262.84	Xenopus laevis Heat shock cognate protein 70
54631889	1816.58	195.95	2430.57	930.41	Eukaryotic translation initiation factor 3 subunit 10
7392205	2476.18	826.05	2425.3	962.92	Fizzy1
530992	1883.54	302.61	2422.7	353.01	Xenopus laevis non-muscle tropomyosin (TM-4)
9368631	1294.28	221.05	2416.13	739.33	Xenopus laevis putative SURF6 protein
56704117	1906.26	95.99	2403.47	881.91	Cu,Zn superoxide dismutase
10756062	1543.14	215.19	2395.87	596.77	Comp60
49899121	1075.08	47.01	2392.57	490.43	Xenopus laevis pantothenate kinase 3
49114986	1172.6	41.55	2380.63	1235.16	Xenopus laevis fibronectin protein

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
987224	3358.16	295.18	2375.67	648.35	Xenopus laevis cytoplasmic polyadenylation element binding protein CPEB
77748122	3250.82	431.4	2364.3	247.4	Xenopus laevis ribosomal protein L4
15991719	1050.08	114.07	2356.67	875.02	Xenopus laevis breast and ovarian cancer susceptibility protein (BRCA1)
11439848	4319.82	255.2	2342.43	420.85	P33 ringo
37904679	1519.22	81	2337.23	249.7	Xenopus laevis NHE3 kinase A regulatory protein 1
49899006	2603.66	199.91	2329	1007.76	Xenopus laevis imitation switch ISWI
33416720	2521.18	244.24	2327.67	376.91	Xenopus laevis L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain
46086649	3453.18	202.46	2302.5	1157.73	HELLS protein
27924336	1560.92	235.02	2285	569.21	Xenopus laevis heterogeneous nuclear ribonucleoprotein A0
108754206	2696.04	97.4	2270.93	328.5	Dendritic cell protein
27735389	4804.7	304.81	2263.13	98.8	Xenopus laevis RAN, member RAS oncogene family
54311174	4035.8	215.81	2262.8	457.55	Xenopus laevis translation initiation factor SUI1
222968	1873.16	246.92	2258.83	382.32	Xenopus laevis RCC1 protein
29483059	1452.18	199.39	2256.5	416.38	67kD laminin receptor precursor
639690	4904.72	85.95	2249.2	863.21	Xenopus laevis HMG-X protein
35505482	1199.32	213.72	2247.2	617.54	Xenopus laevis KH domain-containing transcription factor B3
47123924	1766.58	320.8	2239.57	613.87	Xenopus laevis POU-domain protein
27735475	3787.64	397.79	2234.67	835.11	Xenopus laevis ribosomal protein S14
51258409	2695.86	291.01	2232.83	523.8	Xenopus laevis An2
21952441	2976.6	351.83	2224.9	532.52	Xenopus laevis glutathione s-transferase
27371112	7550.36	442.38	2223	285.65	Xenopus laevis programmed cell death 10
29126977	1098.64	95.55	2209.37	265.98	Xenopus laevis ARP3 actin-related protein 3 homolog
50414763	3036.58	371.72	2206.63	191.22	Xenopus laevis similar to procollagen-proline 4-hydroxylase, beta polypeptide
13641478	1131.48	151.79	2203.73	159.83	Xenopus laevis transcription factor Otx1
92062645	1054	106.84	2185.2	1298.17	Phosphorylase phosphatase
31087961	1691.18	371.1	2178.67	533.55	Xenopus laevis RRM-type RNA-binding protein XSEB4R
28278685	2138.2	400.02	2176.13	796.89	Xenopus laevis TAR DNA binding protein
530992	1566.76	278.92	2174.6	520.18	Xenopus laevis non-muscle tropomyosin (TM-4)
49115171	1074.7	67.03	2146.57	730.2	Xenopus laevis spindle checkpoint protein Bub1
27882616	7132.62	527.11	2140.1	397.83	Xenopus laevis solute carrier family 25 member 5
27882462	1519.48	160.65	2136.63	219.64	Xenopus laevis eukaryotic translation initiation factor 3, subunit 7
27696999	3297.46	162.11	2133.87	592.59	similar to squamous cell carcinoma antigen recognised by T cells
405589	5637.58	357.95	2133.8	1046.52	Frog phospholipase C beta type (PLC1)
27735419	4314.06	446.85	2132.87	219.9	Xenopus laevis ribosomal protein S8

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
49114893	1155.7	49.2	2129.97	360.49	Xenopus laevis sphere organelle protein sph-1
46073908	1180.08	124.04	2096.6	821.17	Similar to RNA-binding region (RNP1, RRM) containing 7
17600331	1350.44	125.62	2092.67	391.84	Proteasome (prosome, macropain) subunit, beta type 1
27881762	2067.94	317.77	2089.8	934.89	Xenopus laevis ribosomal protein S12
28277329	1364.26	53.35	2084.73	217.17	Xenopus laevis Alg5
32450355	2366.72	519.29	2082.03	223.75	Xenopus laevis fructose-1,6-bisphosphatase
27370880	1729.6	183.83	2070.67	199.56	Xenopus laevis RAB11B, member RAS oncogene family
95011377	2091.48	160.5	2068.7	667.74	Activating transcription factor 5
14132773	1379.2	77.08	2046.53	436.45	Xenopus laevis LIS1
27503210	1731.94	163.4	2036.1	320.92	Xenopus laevis eukaryotic translation initiation factor 3, subunit 6 interacting protein
49118222	1162.28	111.18	2032.4	356.27	Xenopus laevis alpha-subunit of G-protein, type G-alpha-i-1
30039224	1193.8	110.16	2028.17	510.44	Xenopus laevis Rae1Gle2 (Rae1Gle2)
27515057	4351.64	403.1	2027	596.01	Histone stem-loop binding protein
38641396	1288.46	91.59	2011.17	919.04	Xenopus laevis nuclear poly(A) binding protein 2
50416288	1154.28	56.49	2006.1	83.93	Xenopus laevis amyloid beta precursor protein (cytoplasmic tail) binding protein 2
46066697	4057.1	300.61	1991.47	472.27	Similar to nuclear RNA helicase
28278092	3467.94	393.84	1982.6	608.09	Xenopus laevis far upstream element (FUSE) binding protein 1
27370868	1623.58	139.95	1977.33	484.76	Xenopus laevis capping protein beta 1
32450638	3405.88	694.01	1967.33	198.32	Xenopus laevis beta-Tubulin
18767723	1338.64	117.78	1966.9	110.4	Xenopus laevis ashwin mRNA
27769400	3266.84	501.13	1966.7	356.7	Xenopus laevis ribosomal protein S1a protein
66910872	3811.08	82.5	1946.37	132.37	Xenopus laevis chromosomal passenger complex protein Dasra A
27769228	1222.68	254.28	1941.5	574.24	Xenopus laevis similar to inosine 5-phosphate dehydrogenase 2
27694693	2003.12	334.77	1937.67	265.41	Xenopus laevis phosphoglycerate kinase 1
54038161	1413.34	219.23	1928.9	169.52	Xenopus laevis TollIL-1 receptor binding protein MyD88
33638097	2428.9	170.46	1923.27	833.03	Xenopus laevis H+ transporting F1 ATP synthase epsilon subunit
27924441	2707.12	298.66	1922.5	1102.55	Xenopus laevis similar to protein regulator of cytokinesis 1-like
1199643	1256.28	101.91	1903.97	282.32	Xenopus laevis alpha(E)-catenin
27515061	2364.34	386.24	1903.8	786.1	Keratin 18
28302202	1741.82	151.05	1893.5	369.97	Xenopus laevis ATP-binding cassette, sub-family E (OABP), member 1
27503417	4521.72	694.05	1884.23	579.01	Xenopus laevis creatine kinase, brain
33642662	1348.28	84.07	1870.73	531.29	SWISNF related, actin dependent regulator of chromatin, subfamily e, member 1
28804565	1910.34	105.82	1860.37	774.91	Xenopus laevis Pumilio
8515824	2117.88	270.67	1858.13	192.56	Xenopus laevis transketolase

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
34099899	2023.74	132.38	1853.87	1096.46	Xenopus laevis transcription factor IIA large subunit-1 (TFIIAab-1)
37295456	1243.16	67.24	1850.7	478.88	Adenylate kinase 2
50414926	2114.52	262.65	1850.47	379.16	Xenopus laevis UDP-N-acteylglucosamine pyrophosphorylase 1
46081230	3041.68	187.42	1849	421.07	Holocytochrome c synthase (cytochrome c heme-lyase)
54311368	1050.22	70.88	1848.77	1093.12	Xenopus laevis scaffold attachment factor A
33417133	2589.48	176.64	1843.7	97.43	Xenopus laevis cytosolic nonspecific dipeptidase
27371023	1603.16	81.32	1831.03	197.6	Xenopus laevis lamin B1
37272452	1897.52	545.86	1830.97	302.34	Similar to ubiquitin C
29836213	2891.84	238.27	1830.83	783.66	Myristoylated alanine rich protein kinase C substrate
27504980	4037.74	191.21	1795.43	387.57	High mobility group HMG-17
50418053	1014.28	152.24	1771.6	462.37	Xenopus laevis sorting nexin associated golgi protein 1
29481254	5575.92	582.02	1764.93	241.16	Thymosin beta 4 peptide
46381768	3673.92	1872.9	1762.37	386.38	Ubiquitin C
27697183	1019.84	110.42	1760.03	547.54	Xenopus laevis protein phosphatase 1G, magnesium-dependent, gamma isoform
27884298	3134.78	468.73	1757.77	34.3	Xenopus laevis xgalectin-VIIa
27503343	3426.32	596.32	1756.53	272.63	Xenopus laevis acidic ribosomal protein P0
27735421	3687.72	700.72	1754.83	215.35	Xenopus laevis maternal G10 transcript
70672229	5637.26	268.76	1754.7	549.56	Xenopus laevis herz03
46379254	3256.66	149.56	1752.23	279.47	Laminin receptor 1
28422241	1836.78	271.67	1747.13	446.22	Xenopus laevis DnaJ (Hsp40) homolog, subfamily B, member 12
28302202	2752.42	222.41	1746.77	509.25	Xenopus laevis ATP-binding cassette, sub-family E (OABP), member 1
50414698	1428.02	170.02	1737.57	354.2	Xenopus laevis TFIIS elongation factor
70895892	1661.08	67.62	1735.17	434.5	Barren homolog
14595659	1111.58	113.83	1733.53	331.52	Xenopus laevis strabismus
32450231	3794.88	560.22	1727.33	802.53	Xenopus laevis cold-inducible RNA binding protein 2
24850051	1036.42	120.82	1726.37	403.51	Xenopus laevis embryonic ectoderm development protein variant 1
27371121	3174.96	680.73	1722.17	329.46	Xenopus laevis similar to GCIP-interacting protein p29
30353794	2325.8	273.25	1721	614.83	Xenopus laevis poly(A) binding protein, cytoplasmic 1
3298594	2453.62	141.21	1716.67	87.03	Xenopus laevis fizzy1
28838484	1711.2	186.52	1715.3	111.09	Xenopus laevis lysyl-tRNA synthetase
76779492	1410.8	174.84	1712.33	225.6	Xenopus laevis cyclin B3
12479117	3321.58	262.09	1710.73	221.07	Mitotic phosphoprotein 22
47124741	1173.46	192.86	1709.43	656.88	Xenopus laevis XLS13B protein (SOX11)
70891327	3966.26	267.84	1704.97	673.5	Eukaryotic translation initiation factor 3 subunit 10

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
3328234	1059.74	111.6	1701.5	289.05	Xenopus laevis 14S cohesin RAD21 subunit mRNA
28436903	2643.14	115.89	1700.97	253.67	Xenopus laevis DEADH (Asp-Glu-Ala-AspHis) box polypeptide 19
70903631	2233.9	334.93	1695.63	379.99	Similar to serinethreonine kinase 12
51949981	1905.22	128.46	1691.73	644.26	Xenopus laevis UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase
3868878	2712.2	240.8	1690.33	187.21	Xenopus laevis Zic-related-2
49899118	2237.5	119.05	1680.27	1181.63	Xenopus laevis zinc finger, A20 domain containing 2
28279873	1604.6	347.72	1671.97	327.05	Xenopus laevis interferon regulatory factor 2 binding protein 2
46249463	3726.5	416.87	1668.27	154.9	Xenopus laevis p33 ringo
50415358	1117.44	59.15	1660.87	300.13	Xenopus laevis RAB3A interacting protein (rabin3)
12476207	2822.28	400.83	1655.5	493.33	C-mos oncogene
42794033	1285.42	37.38	1641.53	61.96	Xenopus laevis death receptor-M1
1041180	2978.46	788.87	1641.23	507.22	Xenopus laevis mRNA for glutamine synthetase
34193963	5887.12	416.22	1638.63	301.34	Xenopus laevis ribosomal protein L27
72372617	1027.78	82.2	1626.53	129.15	CCR4-NOT transcription complex, subunit 10
37290137	1358.58	114.52	1624.77	322.72	Comp48
27694839	2761.04	287.67	1617.8	944.52	Xenopus laevis pyruvate kinase, muscle
58533143	1465.46	115.24	1611.67	229.12	Xenopus laevis heat shock factor 2 protein (HSF2)
56701899	2064.54	185.15	1607.07	328.66	Ribosomal protein S1
67678115	1087.42	140.51	1604.47	689.53	DNA polymerase alpha
27695251	1132.44	51.52	1602.13	49.52	Xenopus laevis ubiquitin-conjugating enzyme E2H
70903389	2671.14	303.7	1598.7	194.89	KH domain RNA-binding protein Sam68
2582136	1033.08	140.64	1596.13	467.43	Xenopus laevis small GTPase Ran binding protein 1
27503907	1341.92	144.98	1595.6	434.06	similar to sirtuin silent mating type information regulation 2 homolog 6
9726371	6693.78	673.3	1579.5	477.88	Variant histone H2A.Zl1
67764067	2559.4	152.07	1578.5	528	Xenopus laevis Fxr1 long isoform variant A
46065620	2377.3	257.92	1572.4	147.48	Cnps01
37194904	2085.14	169.3	1569.57	133.47	Xenopus laevis NTF2-like export factor 1
37962714	2186.94	267.9	1566.33	319.44	Xenopus laevis autosomal recessive hypercholesterolemia beta (ARH beta)
27503447	1234.1	120.67	1565.8	382.75	Xenopus laevis nucleolar GTPase
28958132	7617.78	503.65	1563.5	614.73	Xenopus laevis ribosomal protein S3a
27924330	1157.48	120.65	1562.63	312.45	Xenopus laevis similar to peptidylprolyl isomerase (cyclophilin)-like 2
46075110	2530.24	455.25	1553.67	217.43	H3 histone, family 3B (H3.3B)
70672170	1526.06	380.35	1553.2	344.91	Calreticulin /DEF=Xenopus laevis comp28
37181066	2725.9	286.97	1552.77	100.45	Xenopus laevis p27BBPeIF6

Table 3 - Oocyte mRNAs Present in YY1-Containing mRNPs (continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
28278276	2965.82	219.93	1543.73	175.57	Xenopus laevis similar to t-complex polypeptide 1
27781305	1511.96	361.93	1540.97	269.12	Xenopus laevis Ubiquinol-cytochrome C reductase complex
33585645	2632.66	803.32	1540.37	535.79	Xenopus laevis ribosomal protein S13
11783176	4765.28	492.1	1535.27	730.98	Ribosomal protein L22
49256097	2863.64	152.33	1533.8	289.76	Xenopus laevis similar to ribonucleotide reductase M1
29481798	1702.84	102.49	1523.53	307.69	Basic leucine zipper and W2 domains 1
28436913	2645.42	256.61	1517.53	387.78	Xenopus laevis similar to mannose 6 phosphate receptor binding protein
108752312	2168.48	295.45	1516.63	105.2	Triosephosphate isomerase
27924318	1126.66	115.76	1515.63	63.37	Xenopus laevis translin
37278006	3269.08	205.89	1507.6	470.13	Alpha-tubulin
50417507	1513.76	197.25	1504.07	933.61	Xenopus laevis RNA binding motif protein 5

Table 4 – Oocyte mRNAs Not Present in YY1-containing mRNPs

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
31322353	7591.04	1303.53	16.43	12.98	Xenopus laevis cytochrome b (mitochondrial gene)
5834981	6148.94	1512.03	22.17	20.77	Xenopus laevis NADH dehydrogenase subunit 1 (mitochondrial gene)
5834981	1455.4	633.63	25.47	20.34	Xenopus laevis NADH dehydrogenase subunit 5 (mitochondrial gene)
5834981	6164.38	1134.61	25.47	10.65	Xenopus laevis ATP synthase F0 subunit 8 (mitrochondrial gene)
4096592	1140.78	130.16	33.53	30.52	Xenopus laevis cytochrome c oxidase II (mitochondrial gene)
5834981	3331.8	509.37	38.43	40.76	Xenopus laevis NADH dehydrogenase subunit 3 (mitochondrial gene)
31322381	9483.7	1564.41	42.47	22.98	Xenopus laevis cytochrome b (mitochondrial gene)
31322397	11544	1850.62	43.53	33.43	Xenopus laevis cytochrome b (mitochondrial gene)
50604164	5437.8	835.07	79.63	61.98	Xenopus laevis cDNA clone IMAGE:5506267
5834981	5284.74	491.48	85.77	66.05	Xenopus laevis NADH dehydrogenase subunit 2 (mitochondrial gene)
56789626	1444.82	234.33	91.43	22.55	Xenopus laevis dynein light chain 2
93206491	1000.24	161.7	111.13	30.28	Ribosomal protein L3
57033115	1876.64	144.95	115.13	5.63	Xenopus laevis transcription factor IIA small subunit
28374366	1951.26	2179.09	116.33	48.68	Xenopus laevis similar to Heat shock cognate protein 70
9801986	1835.26	83.12	121.1	41.41	Xenopus laevis DEADSouth RNA helicase
33649910	1007.92	195.14	139.43	26.17	Variant histone H2A.Zl1
31089213	1029.7	571.36	144.53	21.21	Ferritin heavy chain
57033115	1970.28	176.74	149.37	44.99	Xenopus laevis transcription factor IIA small subunit
214020	1080.2	126.92	150.8	29.64	Xenopus laevis beta-catenin mRNA
70672120	8045.04	1714.84	153.83	230.08	Xenopus laevis mito mRNA
38303905	1167.5	42.37	156.23	76.9	Xenopus laevis poly(A) polymerase type 2
27371268	1437.8	123.79	159.9	107.42	Xenopus laevis adaptor-related protein complex AP-3, sigma 1 subunit
27371156	1030.08	58.79	166.87	69.9	Xenopus laevis similar to calcium modulating ligand
29482156	1109.66	68.35	167.37	77.96	Similar to cyclic AMP phosphoprotein, 19 kD
50604168	1125.62	159.62	169.77	119.93	Xenopus laevis protein tyrosine phosphatase type IVA, member 1
28192587	1422.94	160.47	174.07	91.22	Xenopus laevis germes
27503222	1159.98	166.66	181.1	86.5	Xenopus laevis retinoblastoma binding protein 7
46058168	1819.78	249.3	201.47	61.74	Elongation factor 1 alpha, oocyte form
67678145	1316.1	114.3	205.33	70.7	Xenopus laevis importin alpha 1a
31132126	1124.64	152.97	205.57	21.62	HnRNP-E2 protein
49116983	1000.34	82.34	208.83	119.6	Xenopus laevis condensin II non-SMC subunit
27735444	3557.7	478.96	209.8	61.53	Xenopus laevis ribosomal protein L10a
214045	1073.62	111	211	87.23	X.laevis connexin 38
4096592	20389.22	2989.24	212.9	273.84	Xenopus laevis cytochrome c oxidase II (mitochondrial gene)
5834981	13024.02	1374.64	213.9	308.15	Xenopus laevis ATP synthase F0 subunit 6 (mitochondrial gene)

Table 4 – Oocyte mRNAs Not Present in YY1-containing mRNPs (Continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
393276	1075.54	178.83	218.47	89.32	Xenopus laevis protein phosphatase 1-gamma 1
2772903	1135.52	295.91	223.6	39.72	Xenopus laevis DAZ-like protein (dazl)
72377966	1650.92	147.59	223.97	133.39	Gaso01 mRNA
76780127	1350.42	98.01	232.2	64.93	Xenopus laevis RAB5A, member RAS oncogene family
31090329	1568.8	117.27	233.93	32.95	Ribosomal protein S3a
49114953	1043.22	147.83	237	142.4	Xenopus laevis RRM-containing protein SEB-4
27881794	2041.68	165.86	241.17	84.23	Xenopus laevis similar to Nucleophosmin
439107	1872.28	351.26	247.5	105.4	Xenopus laevis maternal protein (wnt-11)
108743257	1025.6	54.8	248.47	144.44	Xenopus laevis LIM-domain-binding protein 1b
29483236	3014.12	425.89	262.3	175.34	Translation initiation factor eIF-5A
967250	1066.24	51.04	270.3	40.07	Xenopus laevis Arf4
58177832	1307.46	151.81	284.23	173.33	Xenopus laevis mis5p
5834981	14712.94	1913.03	288.23	390.57	Xenopus laevis cytochrome b (mitrochondiral gene)
1184106	1464.04	222.98	292.5	156.61	Xenopus laevis DNA replication initiator protein
27370878	1503.78	114.95	294.1	132.85	Xenopus laevis HSPC043 protein
18157369	1357.2	115.03	296.33	178.35	Xenopus laevis X38k protein
28277281	1915.24	82.18	299.43	163.55	Xenopus laevis SMT3
29481199	1140.48	52.27	302.27	154.08	Sorting nexin 2
67678145	2895.62	274.77	304.43	153.14	Xenopus laevis importin alpha 1a
51619913	1165.6	75.75	314.27	105.04	Similar to DnaJ (Hsp40) homolog, subfamily A, member 2
2447042	1098.48	212.95	315.33	120.59	Xenopus laevis xSox7
46329513	2037.32	119.36	319.33	160.42	Xenopus laevis aconitase
32450013	1221.74	277.51	322.4	255.56	Xenopus laevis B-cell translocation gene 1, anti-proliferative
28277279	1040.52	61.16	325.47	144.37	Xenopus laevis programmed cell death 6 protein
46063716	2916.2	200.79	341.2	16.59	Myosin regulatory light chain
57870139	1907.52	205.3	349.2	89.93	Xenopus laevis Ubl5 protein
31451596	1773.24	109.9	354.9	127.43	Eukaryotic translation elongation factor 2
27881770	1021.08	211.08	355.13	114.14	Xenopus laevis monocarboxylate transporter
4324638	2779.02	860.83	355.37	81.73	Xenopus laevis RRM-type RNA-binding protein hermes
50603721	1038.12	164.41	356.37	64.66	Xenopus laevis mitochondrial ribosomal protein L15
27357079	1788.16	556.72	356.57	271.89	Xenopus laevis tumor-associated calcium signal transducer 1
32396219	3302.66	135.06	358.4	60.99	Xenopus laevis velo1
51641002	1617.42	176.33	364.17	117.57	ATPase, H+ transporting, V1 subunit G isoform 1
29485325	1919	296.69	364.97	64.9	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activator theta
93206491	1805.62	289.55	369.43	27.99	Ribosomal protein L3

Table 4 – Oocyte mRNAs Not Present in YY1-containing mRNPs (Continued)

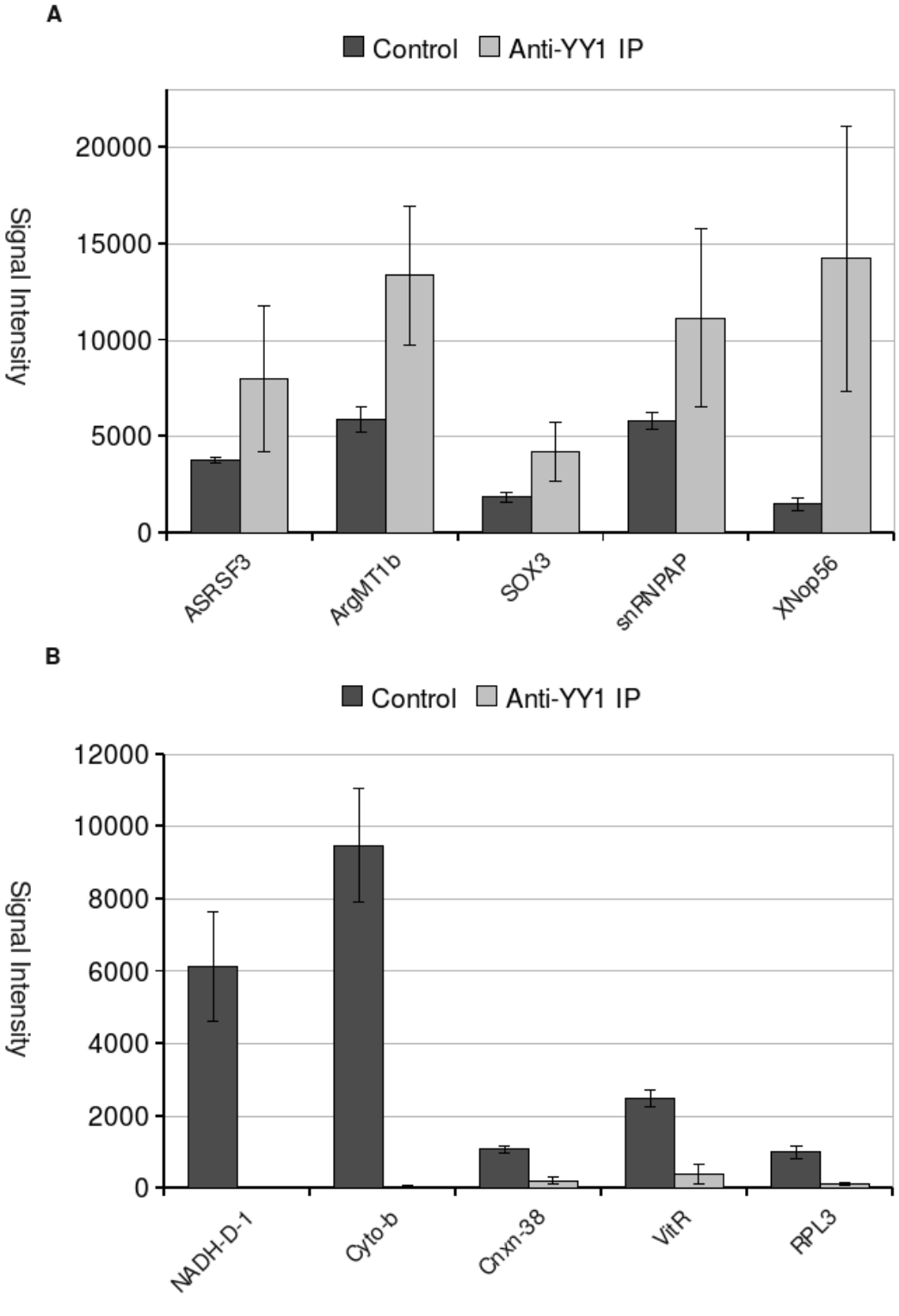
Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
27882459	1472.74	231.51	373.5	193.65	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma 1
54037964	1327.26	138.38	382.43	155.94	Xenopus laevis lamin L(I)
49522130	1103.12	101.21	384.37	47.58	Xenopus laevis heparan sulfate 3-O-sulfotransferase
27469688	1257.78	213.56	390.63	274.42	Xenopus laevis basic leucine zipper and W2 domains 1
95017393	1726.78	174.7	392.97	317.9	P120 isoform 1
77748334	1404.58	155.85	394.23	142.46	Xenopus laevis Aspartate aminotransferase
21072202	1760.62	263.34	395.7	187.41	Chk1 checkpoint kinase
2366772	2471.6	230.17	397.97	286.56	Xenopus laevis vitellogenin receptor
40715950	1475.96	72.17	398.5	52.44	Xenopus laevis kinetochore protein Spc25
49118756	1532.46	71.19	399.57	71.88	Xenopus laevis transcription factor IIA small subunit
1439525	1415.16	88.9	399.9	136.29	Xenopus laevis ubiquitin-like fusion protein
27881781	1493	99.87	402.3	155.49	Xenopus laevis phosphoglucomutase 1
29481798	1054.6	234.27	408.67	22.04	Basic leucine zipper and W2 domains 1
27503188	1575.68	71.08	411.4	374.3	Xenopus laevis integral membrane protein 2B
7698902	1355.82	96.15	411.97	57.33	Similar to splicing factor, argininеserine-rich
46078585	1097.42	97.9	412.17	55.89	Nuclear actin-binding protein chain a
405589	3022.14	722.02	413.1	306.35	Frog phospholipase C beta type (PLC1)
56799122	1206.78	264.8	414.27	84.16	Splicing factor U2AF large chain
28175278	1998.58	187.59	417.53	199.74	Xenopus laevis MAD2 (mitotic arrest deficient, homolog)-like 1
8579206	1996.52	267.96	421.5	94.99	Sec61 gamma subunit
1216408	2579.04	326.88	428.3	158.07	Xenopus laevis zona pellucida glycoprotein homolog (ZPD)
46075445	2099.5	78.93	429.1	139.21	Methyl-CpG binding protein MBD3 long form
28422436	1280.96	294.18	429.8	87.35	Xenopus laevis Serum paraoxonase/aryleterase 2
50416476	1295.8	133.05	436.17	81.47	Xenopus laevis acyl-Coenzyme A dehydrogenase
29483396	1471.36	101.12	437.7	218.87	Nuclear matrix protein NMP200 related to splicing factor PRP19
34784625	1408.14	77.89	453.97	230.46	Xenopus laevis gamma-tubulin interacting protein
46080185	2535.04	42.85	454.6	170.35	Elongation factor 1-alphas
6503024	1759.08	114.12	457.53	164.34	Xenopus laevis SCF complex protein (Skp1)
18139835	1098.62	102.98	461.37	339.7	Williams syndrome transcription factor
28422436	1121.38	207.21	463.8	47.43	Xenopus laevis Serum paraoxonase/arylesterase 2
33585958	1092.28	113.08	471.07	149.73	Xenopus laevis Mitogen-activated protein kinase 2
50418288	1365.92	33.18	471.2	315.02	Xenopus laevis protein phosphatase 4, regulatory subunit 2
46067440	1022.54	84.33	471.7	174.39	Protein phosphatase 4, regulatory subunit 1
31451596	1545.26	194.61	474.53	193.21	Eukaryotic translation elongation factor 2
27371250	1105.32	65.51	475.33	296.64	Xenopus laevis synaptosomal-associated protein, 29kDa

Table 4 – Oocyte mRNAs Not Present in YY1-containing mRNPs (Continued)

Accession	Control		Anti-YY1 IP		Description
	Signal	Std Dev	Signal	Std Dev	
50417950	1212	75.18	476.07	123.13	Xenopus laevis pyruvate dehydrogenase E1 alpha 1
31132610	1599.18	470.18	477.63	136.62	Transmembrane protein 33
68534357	1275.4	39.03	478.03	170.38	Xenopus laevis nucleoporin Nup88A
214034	2205.16	313.23	479.4	334.3	Xenopus laevis cdc2 kinase homologous protein cdc2x1.2)
51635288	1127.86	281.89	480.23	117.99	Translation initiation factor eIF-5A
39983002	1162.86	127.79	480.23	212.13	Xenopus laevis epithelial cell transforming 2 (ECT2)
31131920	1839.8	132.6	487.37	187.84	Unactive progesterone receptor, 23 kD
70904146	2854.9	203.13	493.9	214.94	Similar to ribosomal protein L17
47940271	1062.02	153.23	495.17	356.84	Xenopus laevis mitotic phosphoprotein 140
50603762	1694.62	38.69	496.33	231.36	Xenopus laevis N-ethylmaleimide-sensitive factor beta
24096956	1835.26	57.08	497.9	124.41	Origin recognition complex protein 1

Figure 18 - Comparison of Levels of Selected mRNAs in anti-YY1 IP and Total mRNA. Data from anti-YY1 RIP-CHIP analysis was compared with data obtained from DNA microarray analysis of total oocyte mRNA and used to select five mRNAs with high signal levels in both control and anti-YY1 IP (shown in A); and five mRNAs with high signal levels in control and low signal levels in anti-YY1 IP (shown in B). The average signal level of each mRNA in control RNA is shown in dark grey and the average signal level for each mRNA in anti-YY1 IP RNA is shown in light grey. mRNAs are identified on the x-axis, mean signal intensity is shown on the y-axis. Error bars indicate standard deviation.

Figure 18

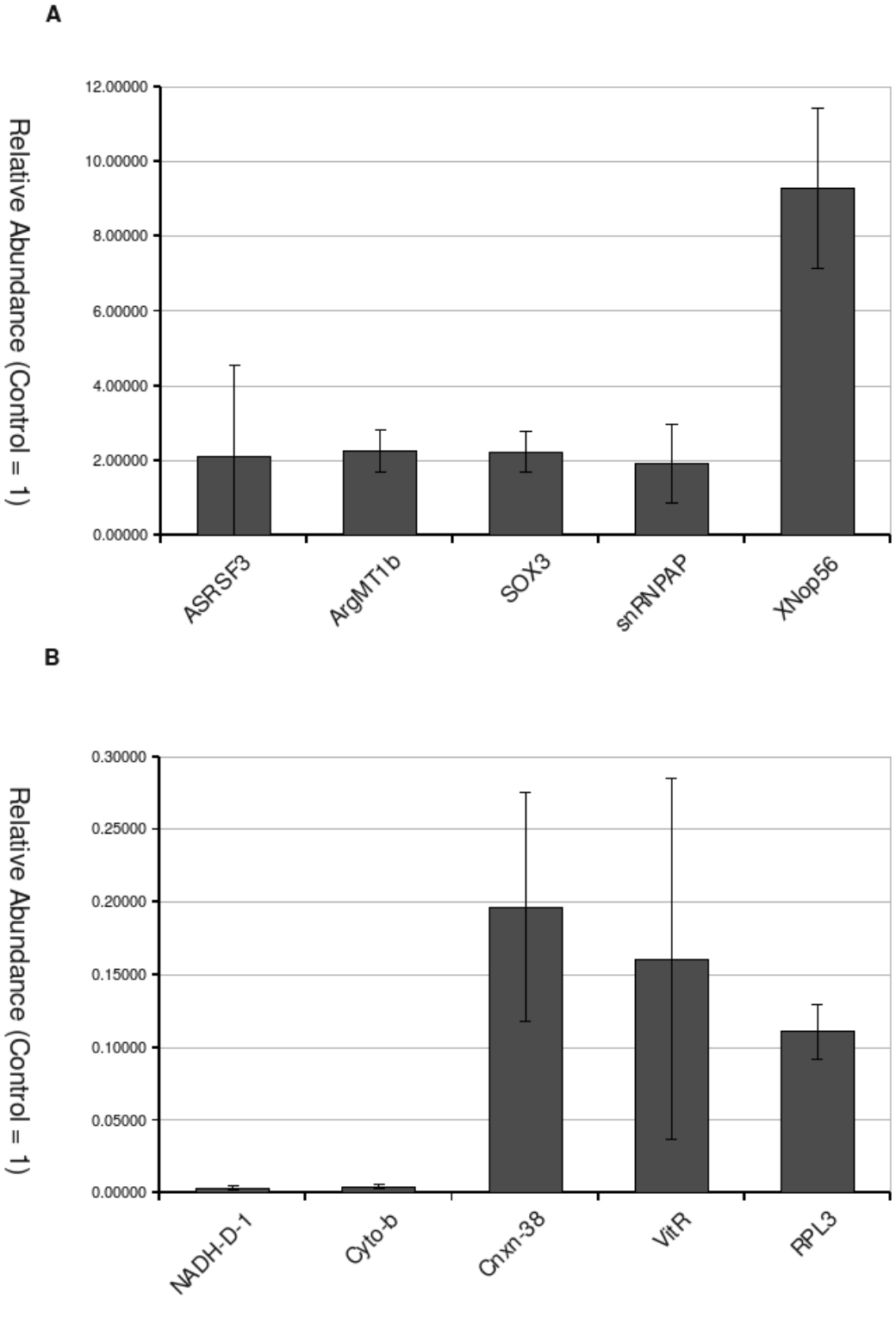

A
Control
Anti-YY1 IP
Signal Intensity
20000
15000
10000
5000
0
ASRSF3
ArgMT1b
SOX3
snRNPAP
XNop56
B
Control
Anti-YY1 IP
Signal Intensity
12000
10000
8000
6000
4000
2000
0
NADH-D-1
Cyto-b
Cnxn-38
VitR
RPL3

3.4.3 Verification of RIP-CHIP analysis by Real-Time PCR – Real-Time PCR was used to verify the accuracy of the RIP-CHIP analysis. Ten mRNAs predicted by RIP-CHIP to be either present or absent in YY1-mRNPs were examined for their relative abundance in YY1-immunoprecipitates versus total oocyte RNA. Average signal levels of these mRNAs in microarrays probed with total RNA and those probed with anti-YY1 IP RNA are shown in Figure 18. Real-Time PCR analysis reactions were conducted on three independently isolated sample replicates of total RNA and YY1 IP RNA. Using the method of Pfaffl *et al.* (Pfaffl, 2001) levels of the selected mRNAs in total oocyte mRNA was set to unity and the average level of the same mRNA in immunoprecipitated samples is expressed in Figure 19 as a fold increase or decrease. Overall, levels of these mRNAs as measured by Real-Time PCR corresponded well with the relative levels obtained by microarray analysis. For example, XNop56, a transcript associated with YY1 in RIP-CHIP was found to be approximately ten times more abundant in YY1-IP RNA versus total oocyte RNA while RPL3, suggested by RIP-CHIP not to associate with YY1 in mRNPs, was only 10 % as abundant in YY1-IP RNA as in total oocyte RNA as assessed by Real-Time PCR (Figure 19). These data substantiate that levels of RNA determined by RIP-CHIP are accurate reflections of actual RNA levels in the samples analyzed. Taken together with the data presented in section 3.4.1, this substantiates the utility of microarray analysis of YY1 immunoprecipitates for determining the mRNA content of YY1-containg mRNPs.

<u>Figure 19</u> - Verification of Gene Chip Results by RT-PCR Analysis of Selected mRNAs.

Levels of selected mRNAs (bottom of panels) were analyzed by quantitative real-time PCR. Values shown are averages of three independent replicate experiments and are displayed relative to the level of the same mRNA in total mRNA. The upper panel (A) shows mRNAs predicted by microarray analysis to be highly enriched in anti-YY1 immunoprecipitates while the lower panel (B) shows mRNAs predicted by microarray analysis to be highly depleted or absent in anti-YY1 immunoprecipitates. Error bars indicate standard error.

Figure 19


A
Relative Abundance (Control = 1)
12.00000
10.00000
8.00000
6.00000
4.00000
2.00000
0.00000
ASRSF3
ArgMT1b
SOX3
snRNPAP
XNop56
B
Relative Abundance (Control = 1)
0.30000
0.25000
0.20000
0.15000
0.10000
0.05000
0.00000
NADH-D-1
Cyto-b
Cnxn-38
VitR
RPL3

3.5 YY1 RNA-Binding Activity is Present in the Cytoplasm of Cultured Cells –

The data presented thus far show that *Xenopus* YY1 possesses high-affinity, substrate-specific RNA binding activity, and that its RNA-binding activity is required for association of the protein with mRNP complexes *in vivo*. Furthermore, DNA microarray analysis demonstrates that YY1 associates with a subset of the mRNAs present in the oocyte. A number of studies have documented the presence of YY1 in the cytoplasm of adult tissue and culture cells in a variety of model systems (Donohoe et al., 1999; Favot et al., 2005; Krippner-Heidenreich et al., 2005; Palko et al., 2004; Rylski et al., 2008), suggesting that YY1 could play a role in mRNA storage in somatic cells. Given the RNA-binding activity of YY1 described here for the first time, it was desirable to perform a comparative analysis of *Xenopus* and Human culture cells, to determine if Human YY1 possesses RNA-binding activity similar to YY1 isolated from oocyte mRNPs. *Xenopus* A6 kidney epithelial cells and Human HepG2 hepatocarcinoma cells were grown in culture and fractionated into nuclear and cytoplasmic extracts. Analysis of these extracts by Western blotting shows that *Xenopus* YY1 protein is present at high levels in the cytoplasm and at moderate levels in the nucleus of A6 cells (Figure 20A). Analysis of HepG2 cells showed the majority of cellular Human YY1 was present in the nuclear fraction with a moderate amount of YY1 present in the cytoplasm (Figure 20A). Western blotting controls with the nuclear protein PCNA showed these findings were not attributable to nuclear contamination of cytoplasmic extracts. EMSA analysis shows that YY1 DNA-binding activity was detectable in both nuclear and cytoplasmic fractions of both A6 and HepG2 cells at levels proportional to the quantities of YY1 protein detected in these cellular compartments by Western blotting (Figure 20A). Next, the cytoplasmic fractions of A6 and HepG2 cells were analyzed by EMSA using U20 RNA probe. A specific YY1-RNA band was detected in both A6 and HepG2 cytoplasmic extracts.

The shifted complex was competed specifically by unlabelled YY1 consensus DNA element but not by mutated consensus DNA (Figure 20B). These data are the first demonstrations of specific YY1-RNA binding activity in the cytoplasm of culture cells, suggesting the ability of YY1 to bind RNA is not restricted to oocytes and early embryos (Ficzycz et al., 2001). Furthermore, the detection of specific YY1 RNA-binding activity in HepG2 cells constitutes the first demonstration of RNA-binding activity by the Human YY1 protein.

Figure 20 - Nucleocytoplasmic Distribution of YY1 in Cultured Cells and Analysis of Cytoplasmic YY1 DNA- and RNA-Binding Activity. (A) Nucleocytoplasmic distribution of YY1 was analyzed in *Xenopus* A6 kidney epithelial cells (A6) and in human hepatocellular carcinoma (HepG2) cells by Western blotting (upper two panels) using 10 μg protein per lane. Antibodies used (indicated at left) were anti-YY1 (YY1) and anti-PCNA (PCNA). Presence of YY1 in the cytoplasm was confirmed by DNA EMSA (lower panel) using 10 μg protein per reaction and 10 nM YY1-consensus DNA probe . The position of the YY1/DNA complex is indicated at left. W, whole cell extract; N, nuclear extract; C, cytoplasmic extract. (B) Cytoplasmic YY1 RNA-binding activity in A6 and HepG2 culture cells was analyzed by EMSA using 20 μg cytoplasmic protein and 10 nM radiolabelled poly-U(20) RNA probe. The position of the YY1/RNA complex is indicated at left. Specificity of the detected band was confirmed by addition of 1 pmol competitor as indicated above the panel. P, probe alone; CL, cytoplasmic lysate; YY1E, double stranded YY1 consensus DNA; YY1M, double stranded YY1 mutant DNA; P, probe alone

Figure 20

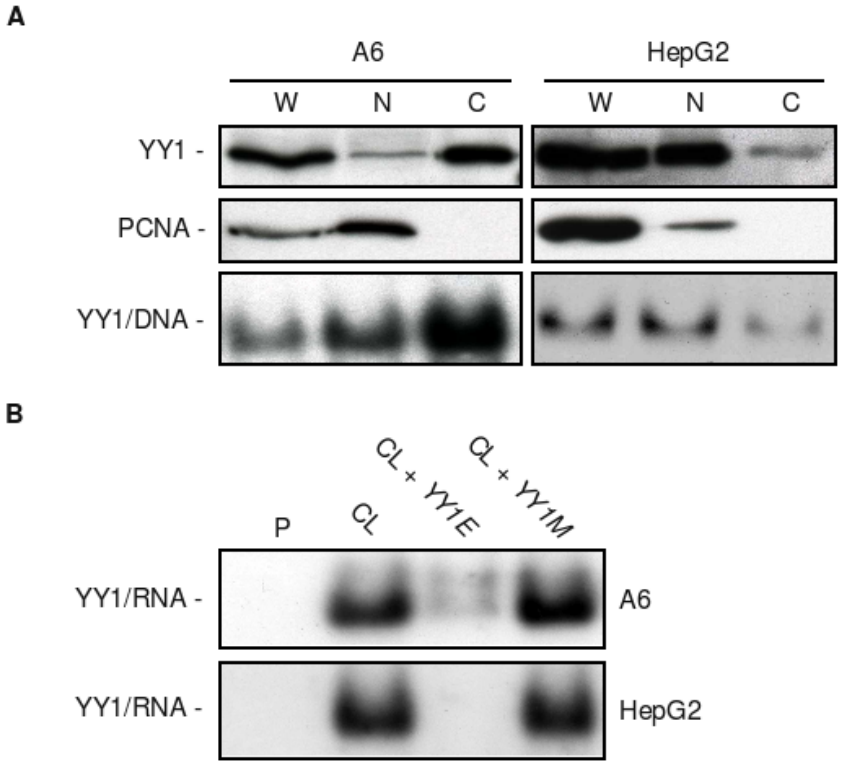

A
A6
HepG2
W
N
C
W
N
C
YY1 -
PCNA -
YY1/DNA -
B
P
CL
CL + YY1E
CL + YY1M
YY1/RNA -
A6
YY1/RNA -
HepG2

Table 5 – Gene Ontology Terms Associated with YY1-bound mRNAs: Molecular Function

GO term	count	GO term	count
protein binding	20	cysteine-type endopeptidase activity	1
ATP binding	14	hydrogen-exporting ATPase activity	1
DNA binding	11	transcription repressor activity	1
zinc ion binding	10	histone binding	1
nucleic acid binding	8	magnesium ion binding	1
RNA binding	7	metalloendopeptidase activity	1
nucleotide binding	6	transcription regulator activity	1
structural constituent of ribosome	5	protein kinase activator activity	1
GTP binding	5	small conjugating protein ligase activity	1
GTPase activity	4	creatine kinase activity	1
unfolded protein binding	4	3'-5'-exoribonuclease activity	1
ATP-dependent helicase activity	3	DNA polymerase processivity factor activity	1
protein serine/threonine kinase activity	3	hydrolase activity	1
structural molecule activity	3	cystic fibrosis transmembrane conductance regulator	1
ubiquitin thiolesterase activity	3	sequence-specific DNA binding	1
ubiquitin protein ligase binding	2	calmodulin binding	1
electron carrier activity	2	oxidoreductase activity	1
binding	2	sodium:dicarboxylate symporter activity	1
protein transporter activity	2	urotensin II receptor activity	1
calcium ion binding	2	protein homodimerization activity	1
ubiquitin-specific protease activity	2	phospholipid binding	1
transcription corepressor activity	2	ubiquitin-protein ligase activity	1
dolichyl-diphosphooligosaccharide-protein glycosylase	1	ligand-dependent nuclear receptor binding	1
G-protein beta/gamma-subunit binding	1	actin binding	1
endoribonuclease activity	1	ubiquitin binding	1
translation initiation factor activity	1	protein kinase C binding	1
ephrin receptor binding	1	Rho guanyl-nucleotide exchange factor activity	1
N-acetyltransferase activity	1	receptor binding	1
signal transducer activity	1	receptor signaling protein tyrosine kinase	1
NADH dehydrogenase (ubiquinone) activity	1	double-stranded DNA binding	1
histone-lysine N-methyltransferase activity	1	protein dimerization activity	1
cyclin binding	1	ligase activity	1
GDP binding	1	single-stranded DNA binding	1
catalytic activity	1	cysteine-type peptidase activity	1
phosphoinositide binding	1	chromatin binding	1

Table 5 – Gene Ontology Terms Associated with YY1-bound mRNAs: Molecular Function

GO term	count
phosphoprotein phosphatase activity	1
calcium-dependent protein kinase regulator	1
transcription factor activity	1
transmembrane receptor protein tyrosine kinase	1
ATPase activity	1
cytokine activity	1
polyribonucleotide nucleotidyltransferase	1
microtubule binding	1
RPTP-like protein binding	1
isocitrate dehydrogenase (NAD+) activity	1
protein C-terminus binding	1
transferase activity	1
poly(A) RNA binding	1
protein disulfide oxidoreductase activity	1
helicase activity	1
mRNA 3'-UTR binding	1
G-quadruplex RNA binding	1
inositol-3-phosphate synthase activity	1
phosphoprotein binding	1
receptor signaling protein activity	1
p53 binding	1
NAD or NADH binding	1
iron-sulfur cluster binding	1
ATPase activity, coupled	1
metal ion binding	1
transcription coactivator activity	1
protein domain specific binding	1

Table 6 - Gene Ontology Terms Associated with YY1-bound mRNAs: Cellular Component

GO term	count	GO term	count
nucleus	20	synaptosome	1
cytoplasm	15	histone pre-mRNA 3'end processing complex	1
integral to membrane	10	microtubule associated complex	1
mitochondrion	6	plasma membrane	1
cytosol	5	proton-transporting V-type ATPase, V1 domain	1
intracellular	4	desmosome	1
mitochondrial inner membrane	4	large ribosomal subunit	1
endoplasmic reticulum membrane	4	chromatin	1
spliceosomal complex	4	costamere	1
nucleosome	4	polysome	1
soluble fraction	3	extracellular space	1
ribosome	3	cell cortex	1
membrane	2	cytoplasmic microtubule	1
nuclear speck	2	cortical cytoskeleton	1
cullin-RING ubiquitin ligase complex	2	transcriptional repressor complex	1
nucleolus	2	germinal vesicle	1
ribonucleoprotein complex	2	condensed chromosome kinetochore	1
respiratory chain	2	calcineurin complex	1
endoplasmic reticulum	2	microtubule organizing center	1
microtubule	2	anaphase-promoting complex	1
Z disc	2	perinuclear region of cytoplasm	1
melanosome	2	microtubule basal body	1
Golgi membrane	2	early endosome membrane	1
transcription factor complex	2	replication fork	1
centrosome	2	endoplasmic reticulum lumen	1
nuclear matrix	2	nuclear envelope	1
signalosome	1	SOSS complex	1
early endosome	1	cyclin-dependent protein kinase holoenzyme	1
nuclear lamina	1	spindle	1
nuclear membrane	1	membrane fraction	1
microsome	1	cytoplasmic vesicle membrane	1
PCNA complex	1	plasma membrane part	1
motile primary cilium	1	photoreceptor connecting cilium	1
neuron projection	1	centriole	1
nuclear pore	1	PcG protein complex	1

Table 6 - Gene Ontology Terms Associated with YY1-bound mRNAs: Cellular Component

GO term	count
CRD-mediated mRNA stability complex	1
Golgi apparatus	1
exosome (RNase complex)	1

Table 7 - Gene Ontology Terms Associated with YY1-bound mRNAs: Biological Process

GO term	count	GO term	count
transcription	10	cell morphogenesis	1
mitosis	5	prenylated protein catabolic process	1
translation	5	central nervous system development	1
nucleosome assembly	4	protein catabolic process	1
cell division	4	mRNA catabolic process	1
regulation of transcription	4	ATP synthesis coupled proton transport	1
ubiquitin-dependent protein catabolic process	4	female meiosis	1
mRNA processing	4	regulation of DNA replication	1
RNA splicing	3	cell redox homeostasis	1
protein folding	3	inositol biosynthetic process	1
protein polymerization	3	negative regulation of translation	1
protein amino acid phosphorylation	3	pro-B cell differentiation	1
microtubule-based movement	3	pituitary gland development	1
hippocampus development	2	protein polyubiquitination	1
protein import into nucleus	2	DNA damage response, signal transduction pathway	1
small GTPase mediated signal transduction	2	response to drug	1
transport	2	eye development	1
electron transport chain	2	protein K11-linked ubiquitination	1
DNA replication	2	positive regulation of axonogenesis	1
negative regulation of transcription	2	nuclear envelope disassembly	1
response to stress	2	axonogenesis	1
synaptic transmission	2	negative regulation of protein amino acid dephosphorylation	1
signal transduction	2	neural tube closure	1
apoptosis	2	T cell differentiation	1
neuron migration	2	neuromuscular process controlling balance	1
regulation of cell cycle	2	lipid catabolic process	1
oxidation reduction	2	histone deubiquitination	1
cell differentiation	2	protein ubiquitination	1
cerebral cortex development	1	regulation of synaptic transmission	1
retrograde axon cargo transport	1	tricarboxylic acid cycle	1
protein transport	1	cell proliferation	1
glucose metabolic process	1	activation of adenylate cyclase activity	1

Table 7 - Gene Ontology Terms Associated with YY1-bound mRNAs: Biological Process

GO term	count	GO term	count
actin cytoskeleton organization	1	ER-associated protein catabolic process	1
heart development	1	protein amino acid autophosphorylation	1
mammary gland epithelial cell proliferation	1	translational initiation	1
rRNA processing	1	response to amphetamine	1
regulation of I-kappaB kinase/NF-kappaB	1	Wnt receptor signaling pathway through beta-catenin	1
cell adhesion	1	DNA recombination	1
positive regulation of glycogen catabolic pathway	1	lung development	1
Wnt receptor signaling pathway	1	lactation	1
RNA processing	1	chromatin assembly or disassembly	1
protein amino acid glycosylation	1	fatty acid oxidation	1
skin development	1	ribosomal protein import into nucleus	1
positive regulation of transcription	1	trophectodermal cellular morphogenesis	1
neuroblast proliferation	1	response to glucocorticoid stimulus	1
transmembrane receptor protein tyrosine kinase	1	peptidyl-tyrosine phosphorylation	1
carbohydrate metabolic process	1	sensory perception of smell	1
G-protein coupled receptor protein signaling	1	regulation of Rho GTPase activity	1
regulation of nucleocytoplasmic transport	1	muscle organ development	1
skeletal muscle fiber development	1	positive regulation of myeloid cell differentiation	1
positive regulation of Wnt receptor signaling	1	base-excision repair, gap-filling	1
positive regulation of hormone secretion	1	protein amino acid O-linked glycosylation	1
dicarboxylic acid transport	1	G2/M transition checkpoint	1
regulation of Rho protein signal transduction	1	positive regulation of gene-specific transciption	1
protein stabilization	1	adult locomotory behavior	1
exocrine pancreas development	1	branching involved in mammary gland duct morphogenesis	1
phospholipid biosynthetic process	1	pigmentation during development	1
acrosome assembly	1	response to caffeine	1
desmosome assembly	1	protein deubiquitination	1
epithelial to mesenchymal transition	1	nuclear envelope organization	1
endoplasmic reticulum unfolded protein response	1	gastrulation	1
protein targeting	1	protein homotetramerization	1
cell-cell adhesion	1	regulation of cell size	1
cell communication	1	ephrin receptor signaling pathway	1

Table 7 - Gene Ontology Terms Associated with YY1-bound mRNAs: Biological Process

GO term	count	GO term	count
calcium ion transport	1	epithelial to mesenchymal transition	1
intracellular protein transport	1	endoplasmic reticulum unfolded protein response	1
G1/S transition of mitotic cell cycle	1	protein targeting	1
double-strand break repair via homologous recombinantion	1	cell-cell adhesion	1
anterior/posterior pattern formation	1		
regulation of transcription, DNA-dependent	1		
camera-type eye morphogenesis	1		
protein amino acid dephosphorylation	1		
positive regulation of protein amino acid phosphorylation	1		
layer formation in the cerebral cortex	1		
dephosphorylation	1		
gamete generation	1		
learning or memory	1		
vesicle transport along microtubule	1		
response to ionizing radiation	1		
DNA repair	1		
response to protein stimulus	1		
regulation of protein metabolic process	1		
positive regulation of gene-specific transcription	1		
phosphocreatine metabolic process	1		
G-protein coupled receptor protein signaling	1		
regulation of nucleocytoplasmic transport	1		
skeletal muscle fiber development	1		
positive regulation of Wnt receptor signaling	1		
positive regulation of hormone secretion	1		
dicarboxylic acid transport	1		
regulation of Rho protein signal transduction	1		
protein stabilization	1		
exocrine pancreas development	1		
phospholipid biosynthetic process	1		
acrosome assembly	1		
desmosome assembly	1		

4. Discussion - The major conclusion of this thesis is that YY1 associates with a subset of maternally transcribed mRNAs via direct RNA-binding activity. Recombinant YY1 was expressed in bacteria and conditions for its efficient isolation and renaturation were empirically determined. The activity of recombinant YY1 was shown to be sensitive to oxidation and the presence of nickel ions. Use of reducing agents during isolation of YY1 and treatment of YY1 with EDTA following IMAC purification were required to achieve full activity of the recombinant protein. YY1 was found to have a magnesium- and zinc-dependent RNA-binding activity with some sequence specificity toward U-rich sequences, and a minimal requirement of 11 consecutive nucleotides for RNA-binding. This novel biochemical activity of YY1, described here and in a recent *Journal of Biological Chemistry* article for the first time (Belak and Ovsenek, 2007), was shown to be required for association of YY1 with mRNPs in *Xenopus* oocytes. Additionally, YY1-RNA interactions were highly stable. RNA-immunoprecipitation/DNA microarray (RIP-CHIP) analysis was used to analyze the population of oocyte mRNAs associated with YY1 in mRNPs. It was shown that YY1 associates with a highly varied population of mRNAs that appear to be utilized later in development. There were a number of maternal transcripts that were not detected in the YY1-mRNP pool, encoding proteins such as Vitellogenin Receptor, Zona Pellucida Glycoprotein Homologue, and oocyte-specific Elongation Factor-1α isoform known to be translated specifically in the oocyte. In addition, transcripts from the mitochondrial genome were not detected in the YY1-mRNP pool, a finding consistent with the efficacy and fidelity of the RIP-CHIP methodology. Of all known mRNP constituent proteins, YY1 is the first for which the *in vivo* mRNA substrates have been determined. Finally, YY1 protein was shown through biochemical assays to be present in the cytoplasm of both *Xenopus* and human culture cells, and a specific YY1 RNA-binding activity

was detected in adult cells of human and *Xenopus* origin. Taken together, these data offer considerable support for a biological role for YY1 outside transcriptional regulation, and provide some insight into the general structure of mRNPs.

4.1 Purification and Renaturation of Recombinant YY1 – Immobilized metal affinity chromatography is widely used for the production of recombinant proteins for a variety of applications. Basic IMAC protocols are often sufficient for production of recombinant proteins either because subsequent applications do not require high specific activity or target proteins assume their native conformations under standard conditions (Porath, 1992). In the case of YY1, however, there was a requirement for extensive optimization of the IMAC procedure to achieve high specific activities. For applications such as analysis of protein-nucleic acid binding affinities it is especially important to pay close attention to the conditions utilized in production of the recombinant protein in question (Block et al., 2009). A disciplined approach to the design of IMAC protocols therefore ensures the integrity and accuracy of *in vitro* experimentation aimed at determining specific protein activities, in this case, the RNA-binding activity of YY1. To this end it was necessary that the conditions used in the preparation of recombinant YY1 resulted in a product that was highly purified, is in the native conformation, and free of contaminants that could interfere with or inhibit protein activity. In general IMAC applications, the effects of protein oxidation and the presence of residual metal ions from immobilized metal affinity chromatography are often overlooked (Belew et al., 1987; Block et al., 2009; Chaga, 2001; Esfandiar et al., 2010; Gaberc-Porekar and Menart, 2001; Porath, 1992; Zhu et al., 2005). Careful control and analysis of the procedures and reagents used in production of recombinant YY1 used in this study resulted in greater reproducibility, higher specific activities, and results which more accurately reflected protein activities *in vivo* (Block et al., 2009; Chaga, 2001;

Gaberc-Porekar and Menart, 2001; Porath, 1992; Smith et al., 1988).

Here, the parameters of protein purification using IMAC are discussed in some detail. A suitable lysis buffer has three characteristics: It must efficiently lyse the bacterial cells, it must provide an environment in which the target protein is soluble and stable, and finally, must be compatible with subsequent chromatography steps. Initial work therefore focused on comparing the effectiveness of different lysis methods for preparation of YY1, involving comparison of buffers with lysozyme and various denaturants. Lysozyme is a standard reagent used to degrade the cell wall of *E. coli* and facilitate lysis of bacterial cells for isolation of recombinant proteins (Birdsell and Cota-Robles, 1967; Block et al., 2009; Zinder and Arndt, 1956). Lysozyme is active in buffers which are iso-osmotic with respect to bacterial cells (Birdsell and Cota-Robles, 1967; Dickman and Proctor, 1952), therefore, utilizing lysozyme offers the advantage of isolation of proteins under native or near-native conditions, which would facilitate analysis of YY1. One disadvantage of lysozyme is diminished protein activity under reducing conditions (Abraham, 1939; Dickman and Proctor, 1952; Jolles and Jauregui-Aolles, 1963). Given the sensitivity of YY1 to oxidation shown in Figures 2B and 4C, the inclusion of reducing agents is desirable, but precludes the use of lysozyme. Furthermore, Figure 2A demonstrates that when bacterial cells expressing YY1 were subjected to lysis by lysozyme, little of the YY1 protein was present in the soluble fraction. Since the activity of the lysozyme preparation used in this work was verified, it is concluded that formation of YY1 inclusion bodies in bacterial cells is the most likely explanation for low YY1 solubility in non-denaturing lysis buffers (Razeghifard, 2004).

Generally, eukaryotic proteins are not folded efficiently into their native conformations in prokaryotic expression systems causing eukaryotic proteins isolated after prokaryotic expression to exhibit low specific activity relative to these proteins in their

endogenous cellular environments (Chang et al., 2005). Therefore it is often preferable to isolate recombinant proteins in the presence of strong denaturing agents and to subsequently renature them. Guanidium offers the advantage over urea of higher solubility at lower temperatures, allowing protein isolation to be performed at low temperature (4 °C), however, the disadvantage of insoluble complex formation with SDS necessitates its removal from recombinant proteins prior to SDS-PAGE analysis. This introduces the cumbersome additional step of ethanol precipitation (guanidium hydrochloride is insoluble in acetone), which reduces the ease of sample analysis, and is eliminated by the effectiveness of 8 M urea in solubilising recombinant YY1 (Figure 2). The main drawback of urea for protein purification by IMAC protocols is reduced solubility at the lower temperatures frequently used in IMAC steps. This problem was overcome in two ways: First, after lysis, buffers were diluted to 4 M urea prior to chromatography, so that variations in ambient temperature down to ~10 °C were tolerable. Second, YY1 was stable for at least 72 h at temperatures up to 37 °C in buffers containing 4 M urea and 1 mM 2-mercaptoethanol (Figure 2B). In conclusion, lysis buffers containing 8 M urea are ideal for YY1 isolation due to effective lysis of bacterial cells, solubilisation of recombinant protein, and compatibility with added reducing agents and subsequent IMAC steps.

It was shown in Figures 2B and 4C that YY1 is sensitive to oxidation. Formation of both intermolecular and intramolecular disulfide bonds in YY1 would reduce specific activity of the purified protein, therefore use of reducing conditions in all protein isolation steps is advisable. This is particularly relevant when experiments measuring binding affinities of recombinant proteins are undertaken since protein preparations with abnormally low specific activities would yield inaccurate affinity values.

The selection of reducing agent is influenced by several factors. Dithiothreitol (DTT)

is commonly used at concentrations of 1 mM to prevent protein oxidation in solution, however it is unstable in aqueous solutions at pH exceeding 7.0 at temperatures above freezing (Getz et al., 1999) making it particularly inappropriate for inclusion in wash and elution buffers stored at room temperature. Due to its ready availability and stability in aqueous solution, 2-mercaptoethanol was used to maintain reducing conditions in lysis, wash, elution, and renaturation buffers. The data in Figure 2B and 4C show that 2-mercaptoethanol at concentrations of 1 mM effectively prevented oxidative aggregation of proteins in lysates. The relatively new reducing agent tris(2-carboxyethyl)phosphine (TCEP) was not evaluated in this study. TCEP has a number of potential advantages such as stability in aqueous solution and non-volatility (Getz et al., 1999). TCEP does not complex or reduce nickel ions bound to NTA-agarose and so is compatible with IMAC resins (Bergendahl et al., 2002). Given the higher cost of TCEP and the demonstrated effectiveness of 2-mercaptoethanol, it is concluded that 2-mercaptoethanol is satisfactory for the current application.

There have been a number of reports of efficient on-column renaturation of various recombinant proteins using IMAC (Esfandiar et al., 2010; Li et al., 2004; Liu et al., 2007; Razeghifard, 2004; Zhu et al., 2005). The concept of on-column renaturation is intriguing and should be considered in many applications due the simplicity of direct elution of active protein. In the present example, columns with bound YY1 were washed and eluted under conditions that would presumably favour renaturation and acquisition of activity. However, this simple procedure resulted in suboptimal YY1 nucleic acid binding activity even after extensive removal of imidazole.

It was found that residual nickel ions from IMAC chromatography had a strong inhibitory effect on YY1 nucleic acid binding activity (Figure 4C). For these reasons, it was

necessary to supplement eluted YY1 with guanidium isothiocyanate and EDTA and to perform renaturation by exchange into buffers containing zinc and magnesium. The data in Figure 4A and B show that YY1 was renatured effectively using these conditions. Collectively, the data demonstrate the importance of both redox and metal ion effects in the preparation of the recombinant YY1 used in this study for *in vitro* analyses.

4.2 RNA-Binding Activity of YY1 - Bacterially expressed, highly purified, recombinant YY1 was used for *in vitro* analysis of RNA-binding activity with a variety of probes (Figures 6 - 10), and the results were confirmed using endogenous YY1 isolated from oocyte mRNPs (Figure 13). These findings confirm the mechanism by which cytoplasmic YY1 associates with maternally derived mRNA molecules in mRNP complexes is through direct RNA binding activity. The major conclusions arising from this data are that YY1 has sequence specific RNA-binding activity for U-rich single stranded and A:U duplex RNA; and that YY1 does not appear to associate with RNA ends, but with internal regions of maternal transcript (Figures 8 – 10).

YY1 displayed highest affinity for single stranded poly-U RNA (Table 2), on the same order of affinity as for its DNA consensus site (Houbaviy and Burley, 2001; Houbaviy et al., 1996; Momoeda et al., 1995). YY1 displayed sequence specificity in its RNA-binding characteristics, with high affinity for A:U duplexes and single-stranded U-rich regions. It is hypothesized that these sequences are likely targets of YY1 in mRNPs *in vivo*. Binding to several single-stranded substrates other than U_{20} was also observed, however, intramolecular A, G, or C residues significantly reduced or abolished binding for U-rich regions in both single and double stranded substrates (Figure 10, Table 2). Low affinity binding was observed for several substrates, with affinities substantially lower than for the DNA consensus (Figure 10, Table 2).

No affinity was observed for RNA sequences such as C_{20}, $(AC)_{10}$, or any of the duplexes with the exception of A:U. Furthermore, very low affinity was observed for RNA probes containing YY1 DNA consensus sequences (Figure 10). It is therefore unlikely that the high-affinity RNA-binding activities observed for U_{20} and A:U observed in this study is solely the result of non-specific or backbone interactions between YY1 and these RNA probes. The data taken together demonstrate sequence specificity in YY1 RNA binding. It therefore appears that YY1 recognizes internal regions of mRNAs, perhaps at U-rich or A:U-rich duplex regions within mRNAs (Figure 10). This is supported by data showing YY1 did not bind to the 7-mGpppG 5'-cap structure (Figure 8, Table 2), or single stranded poly-A probes which would mimic the poly-A tail of maternal mRNAs (Figure 10, Table 2).

A number of experiments showed that YY1-RNA interactions are highly stable (Figure 11) which would be consistent with the hypothesized role for YY1 in long-term RNA storage through the protracted duration of oogenesis and into embryonic development. High-affinity RNA binding activity of YY1 was observed at physiological temperatures, in >0.4 M sodium chloride, and in the presence of non-ionic detergents (Figure 11). These data are consistent with endogenous YY1 exhibiting RNA-binding activity under the physiological conditions of the *Xenopus* oocyte and further support the hypothesis that the observed RNA-binding activity of YY1 is present *in vivo* and underlies the mechanism of association of YY1 with mRNPs. Indeed, interaction of endogenous YY1 with mRNPs was destabilized at similar concentrations of sodium chloride as those that destabilized interaction of recombinant YY1 with RNA *in vitro.* In any event, the high affinity observed in YY1-RNA interactions clearly implies a relevant biological activity, and could easily account for the presence of YY1 in mRNPs.

The substrate specificity of YY1 strongly suggests that RNA recognition occurs by a

different mechanism than DNA recognition since YY1 displayed very low affinity for single- or double-stranded RNA oligonucleotides containing sequences homologous to the DNA-binding site. Of all known zinc finger RNA-binding proteins, the precise mode of RNA-interaction has only been elucidated for three proteins. These include the *Xenopus* C2H2 zinc finger proteins TFIIIA and ZFa, and the human CCCH zinc finger protein TS11d (Hall, 2005; Lu et al., 2003; Moller et al., 2005). Whereas the mode of DNA recognition by zinc finger proteins invariably involves recognition helix/major groove interactions, the same general principles do not apply to RNA recognition (Brown, 2005). The RNA-binding zinc fingers of TFIIIA make specific contacts with RNA bases in highly structured helical and non-helical regions, and it binds a double-stranded region of the 5S RNA with high sequence specificity (Hall, 2005). ZFa, in contrast, binds double stranded RNA with little apparent sequence specificity, primarily through interaction with the RNA backbone (Moller et al., 2005). YY1 displays less apparent RNA sequence specificity than TFIIIA but more than ZFa (Figure 10, Table 2). YY1 displays high affinity for a single stranded substrate, implying divergence in the mode of RNA recognition between C2H2 proteins. Interestingly, YY1 behaviour more closely resembles the CCCH zinc finger protein TS11d. TS11d has high affinity for AU-rich duplex RNA and the solution structure of this protein in complex with RNA has been determined (Hall, 2005). Sequence specificity of TS11d is apparently mediated entirely by peptide backbone contacts with Watson-Crick base pairs in helical RNA. Given the capacity of YY1 to bind single stranded substrates (poly-U) it would appear there is considerable variability in the mechanisms by which zinc finger proteins recognize RNA.

The present study constitutes the most comprehensive analysis of the RNA-binding activity of any of the known *Xenopus* mRNP proteins. Despite the absence of detailed structural

or affinity analyses, some of the general RNA-binding characteristics of Xp54, FRGY2a/b, and Rap55 have been established and previous work provides a context in which to consider the observed characteristics of YY1. Xp54 is a double-stranded RNA-binding DEAD-box protein that displays ATP-dependent RNA-helicase activity when phosphorylated (Ladomery et al., 1997; Minshall et al., 2001). FRGY2A/B are cold shock domain proteins that associate with single-stranded regions of RNA (Marello et al., 1992). Like YY1, FRGY2A/B possesses dual functional roles in transcriptional regulation and RNA-binding in mRNPs. Some reports indicate FRGY2A/B have sequence specificity toward RNA substrates with sequences similar to the FRGY2A/B DNA consensus site (Bouvet et al., 1995; Matsumoto et al., 1996). This behaviour is in contrast to the findings with YY1, which did not show appreciable affinity for RNAs containing the DNA consensus site (Figure 9). Rap55 possesses an RGG RNA-binding domain responsible for generalized, non-specific interaction with RNA; and there is some experimental evidence to suggest it plays a role in translational repression of bound transcripts (Lieb et al., 1998; Marnef et al., 2009; Tanaka et al., 2006). YY1, in comparison, is a multifunctional protein containing four C-terminal zinc fingers (Shi et al., 1997). YY1 differs from other mRNP proteins in displaying significant RNA sequence specificity and its ability to associate with both single and double stranded RNA. The functional consequences of these differences remain to be determined.

The major mRNP proteins had previously been shown to be recruited to maternal mRNA molecules destined for long term storage in the oocyte (Ladomery et al., 1997; Matsumoto et al., 1996; Moore, 2005; Yurkova and Murray, 1997), as is shown here to be true for YY1. It is generally believed that these proteins are universal constituents of mRNPs with potential roles in translational regulation (Marello et al., 1992; Marnef et al., 2009; Matsumoto et al., 1996;

Minshall and Standart, 2004; Minshall et al., 2001; Tanaka et al., 2006; Yurkova and Murray, 1997).

The mechanisms by which mRNAs are selectively recruited from the mRNP pool for translation at the correct developmental stage have not yet been determined. Therefore, the next logical step in this analysis was to identify mRNAs associated with YY1 in oocytes (see below). The major finding of the data presented in Figures 5 to 15, specifically that YY1 is able to associate directly with RNA and that this RNA-binding activity is the mechanism by which YY1 is incorporated into mRNPs, is an important step toward determining its precise biochemical role in early development.

4.3 Procedural, Statistical, and Bioinformatic Considerations for RIP-CHIP

Analysis of YY1-mRNPs - In order to determine the population of oocyte mRNA molecules bound by YY1, an RNA-immunoprecipitation/DNA microarray (RIP-CHIP) approach was used. In this approach, oocyte lysates were prepared under conditions that preserve the integrity of mRNPs. This lysate is then used in immunoprecipitation reactions with anti-YY1 antibody. This facilitated isolation of mRNP-YY1 along with the associated mRNAs. The isolated mRNA was amplified by two rounds of reverse transcription followed by *in vitro* transcription. The cRNA (copy RNA) was labelled by inclusion of biotin-UTP in the last *in vitro* transcription step. Total oocyte RNA was isolated and labelled in a single round of reverse transcription followed by *in vitro* transcription in the presence of labelled UTP. By hybridization of the labelled cRNA to a *Xenopus laevis* whole-genome DNA microarray it was possible to determine the subset of mRNAs present in both total oocyte and in YY1-IP samples. The assumption regarding fidelity of this assay was that only mRNAs bound to YY1 would be isolated during anti-YY1 immunoprecipitation and detected on the microarray hybridized with YY1-IP RNA. This assumption was then verified by Real-Time PCR of reverse transcribed mRNA isolated by anti-YY1 immunoprecipitation. The working hypotheses were that YY1 would associate with a subset a subset of maternally transcribed mRNA molecules, and that therefore mRNPs are heterogeneous with respect to their mRNA content.

This constitutes a novel application of DNA microarrays and as such, algorithmic methods for analysis of the resulting data currently do not exist. A comprehensive discussion of the computer and statistical algorithms required for such analysis is out of the scope of the current work. Therefore, a conservative initial categorical analysis was applied to the data: mRNAs with high signal intensities in total RNA samples that were substantially enriched in

YY1-IP RNA samples were presumed to be boundto YY1 *in vivo* while mRNAs which possessed high signal levels in total RNA samples but substantially lower signal values in YY1-IP RNA samples were presumed not to be bound by YY1 *in vivo*. This conservative analysis sharply reduced the chances of false positives but also likely resulted in false negatives; i.e. the elimination of many *bona fide* YY1 mRNA substrates from the list of transcripts associated with YY1. This is due to the fact that mRNAs with low signal levels in total oocyte RNA and high signal levels in YY1-IP RNA would be classified as "not associated with YY1" even though this disparity almost certainly represents a significant enrichment of these mRNAs in YY1 IPs. However, in the absence of rigorous statistical analysis of the data it would not be possible to state with confidence that these mRNAs were in fact present in the total oocyte pool, and that their high signal level in anti-YY1 immunoprecipitates was not anomalous.

As mentioned above, the potential existed for assignment of false positives. The expected relative abundance for an unbound mRNA between total oocyte mRNA and immunoprecipitation reactions is approximately 10^{-6} (i.e. an unbound RNA with signal level 100,000 in total RNA would be expected to have a signal level of 0.1 in IP material), however, the two step amplification procedure used could increase this to as much as 10^{-2}. Examination of the data presented in Table 3 reveals that among the top mRNAs classified as associating with YY1, virtually all were present at substantially higher levels in anti-YY1 IPs than in total RNA, as would be expected if they were enriched by immunoprecipitation and amplification steps. This makes it unlikely that any false positives were generated by the classification scheme described above. Furthermore, examination and comparison of the data in Tables 3 and 4 (comparative signal levels read from the hybridized array) show that while mRNAs listed as associated with YY1 were invariably enriched in anti-YY1 IPs (Table 3), those listed as not

associated (Table 4) were present in YY1 IPs at levels < 30% of their levels in total RNA. It is extremely unlikely that any phenomenon other than direct interaction with YY1 and immunoprecipitation with anti-YY1 antibody could result in some RNAs being enriched in YY1 IPs to levels in excess of ten times that in total RNA whilst others are present at less than 10% of that in total RNA. Confirmation of this reasoning was obtained by Real-Time PCR analysis of immunoprecipitated RNA, which confirmed relative levels of mRNA determined by RIP-CHIP analysis (Figure 19). Ultimately, application of advanced bioinformatic analysis to the data will address important questions, such as determination of common structural features of YY1 bound mRNAs, and better classification of these mRNAs into functional groups. However, the data discussed above show the current analysis is sufficient to conclude that YY1 associates with a subset of maternally transcribed mRNAs; in particular, mRNAs which are not translated in the oocyte and are recruited later in development, consistent with the hypothesized roles in mRNA storage and regulated translation.

4.4 RIP-CHIP analysis of YY1-Containing mRNPs –YY1-mRNPs (Table 3) contained mRNA transcripts encoding proteins with a vast array of cellular functions. Transcripts encoding ribosomal proteins, cell cycle control, signalling, and histone proteins comprised the most prominent groupings, representing ~14% of the transcripts identified in the YY1-mRNP pool. The remaining ~86% of transcripts encompassed an extremely varied population. A number of transcripts encoding ribosomal proteins were observed including L4, L4b, L5, L7a, L8, L9, L12, L13, L13a, L15, L19, L22, L24, L27, L30, L35a, S1, S1a, S6, S8, S9, S10, S11, S12, S13, S14, S19, S21, and S27. This suggests a possible role for YY1 in regulation of ribosome biosynthesis during embryogenesis. This is particularly interesting since YY1 is involved in transcriptional regulation of ribosomal protein expression (De Rinaldis et al., 1998;

Perry, 2005; Pisaneschi et al., 1994; Voronina et al., 2008), suggesting YY1 regulates ribosome biosynthesis at both a transcriptional and a translational level. YY1 also associated with mRNAs for a number of Cyclin mRNAs, including Cyclins A1, A2, B1, B2, B3, B4, B5, and E3; as well as transcripts coding for important cell cycle control proteins such as Spindle Checkpoint protein Bub1, Ras, Rab11B, C-mos, Cdc25A, Cdk9, and Cdc45. These observations correlate with data indicating YY1 plays an important transcriptional role in cell cycle control (Gordon et al., 2006; Palko et al., 2004; Petkova et al., 2001; Shi et al., 1997; Sui et al., 2004), and suggest that, at least in the oocyte and early embryo, YY1 may contribute to cell cycle regulation through non-transcriptional mechanisms. YY1 also bound to several transcripts for histone proteins such as H4, H1A, H1.B4, H3.3A, H3.3B, and H2A.z, and again, this correlates with data showing regulation of histone expression by YY1 at a transcriptional level (Eliassen et al., 1998; Horvath et al., 2003; Last et al., 1999; van der Meijden et al., 1998; Wu and Lee, 2001). Transcripts encoding protein products involved in intracellular signalling cascades were also associated with YY1, including the Wee1A kinase, Casein Kinase Iε, Polo-like kinase, brain-specific Casein Kinase, and Protein Phosphatase 1G-γ, and Insulin-like Growth Factor Receptor 1β.

YY1 associates with many tissue- and organ- specific transcripts which are not expressed in the oocyte or during the very early stages of embryogenesis, supporting the hypothesis that these transcripts are stored and selectively utilized later in development. However, YY1 also bound many transcripts associated with basic cellular functions, including transcripts for cytoskeletal proteins such as Alpha-/Beta-tubulin, components of basic metabolic pathways such as Glutamine Synthetase, Citrate Synthetase, and proteins of the mitochondrial ATP Synthetase complex. These data make it impossible to speculate as to whether YY1

associates (and possibly regulates) the targeted translation of specific stored mRNAs in specific tissues or is instead a general component of mRNPs which associates with many mRNAs which are utilized during subsequent embryonic development.

The data acquired from microarray analysis of YY1-associated mRNAs revealed several trends. Among the 119 transcripts not associated with YY1-mRNPs, 12 were products of the mitochondrial genome, while, importantly, no mitochondrial transcripts were found among transcripts identified as components of YY1-mRNPs. This finding is consistent with previous work in *Xenopus* oocytes showing that only transcripts of nuclear origin are targeted for storage in mRNP particles (Braddock et al., 1994; Minshall and Standart, 2004; Smillie and Sommerville, 2002; Smith and Richter, 1985; Weston and Sommerville, 2006). Also notable among the pool of mRNAs not associated with YY1 were transcripts for proteins expressed specifically in the oocyte, including Vitellogenin Receptor, the *Xenopus* Zona Pellucida Glycoprotein homologue, Synaptosomal-associated Protein, Beta-Catenin, oocyte-specific Elongation Factor-1α isoform, Condensin-II non-SMC subunit, Wnt-11, and Sec61γ (Table 4). The absence of these transcripts in anti-YY1 immunoprecipitates is consistent with the view that mRNPs store mRNAs required for later development. Since the above transcripts are actively translated to provide protein products required in the oocyte, it fits with accepted views of mRNP function that they be excluded from the mRNP pool, specifically, that mRNPs contain mRNAs destined for long-term storage rather than immediate translation. The observation that mitochondrial transcripts and mRNAs for proteins expressed abundantly in the oocyte are not present in YY1 immunoprecipitates provides convincing evidence that the RIP-CHIP analysis undertaken here is able to accurately differentiate transcripts associated with YY1-mRNPs from those that are not.

The major finding of this study remains the discovery of YY1 RNA-binding activity and its role in YY1-mRNP association. It is clear that YY1 associates with a subset of the maternal mRNA pool, specifically, nuclear transcripts which are not expressed in the oocyte but are instead stored for use later in development. The RIP-CHIP data presented here are in need of further statistical refinement before they can be used to determine a definitive profile of transcripts in the YY1-mRNP pool. However, viewed with some conservatism these data are useful in guiding testable hypotheses with regard to the biological function of YY1-RNA complexes. To determine a biological function means that one must show that a particular protein or its activity is required for a particular biological process. This evidence is lacking in the case of YY1, since neither RNA-binding nor mRNP association of YY1 has been definitively linked to any particular biological process. This issue is discussed with the understanding that neither the RNA-binding nor mRNP-association of any of the known mRNP proteins has been tied to any biological process. A review of the literature reveals that while the biochemical properties of these proteins have been studied in relative detail, their role in development is largely unknown. Although the literature vaguely describes the role of these proteins as "mRNA storage" or "regulators of translation" neither of these functions has been demonstrated *in vivo* (Bouvet et al., 1995; Bouvet and Wolffe, 1994; Braddock et al., 1994; Dreyfuss et al., 2002; Graumann and Marahiel, 1998; Ladomery et al., 1997; Lieb et al., 1998; Marello et al., 1992; Marnef et al., 2009; Matsumoto et al., 2000; Matsumoto et al., 1996; Minshall and Standart, 2004; Minshall et al., 2001; Moore, 2005; Smillie and Sommerville, 2002; Standart and Minshall, 2008; Tanaka et al., 2006; Weston and Sommerville, 2006; Yang et al., 2006; Yurkova and Murray, 1997). It is clear, however, that the oocyte contains numerous mRNAs that code for proteins utilized in later development (Table 3), and since these mRNAs are associated with

mRNPs it stands to reason that these complexes must have a role in their selective recruitment at the appropriate developmental stage and location. As with other mRNP proteins, precise determination of this role has heretofore been hampered by a lack of knowledge as to what biological processes one should look for as evidence of a role of YY1 RNA-binding. Determination of the mRNA substrates of YY1, described here for the first time, will facilitate examination of the role of YY1-mRNPs in specific cellular processes and in distinct developmental events.

5. Future Work – Ongoing bioinformatic analysis of the RIP-CHIP data will determine common structural, sequence, and functional characteristics among YY1-associated mRNA molecules. The data presented here allow testable hypotheses to be formulated regarding the role of YY1 RNA-binding in mRNPs as related to key developmental and cellular processes. Suggested future work would involve a number of experimental approaches. Using immunoprecipitations and Real-Time PCR, the association of selected mRNAs with YY1 can be examined at specific stages of embryonic development. This will determine if and when tissue-specific mRNAs are mobilized from the mRNP pool. These observations can be correlated with expression of the associated protein products by Western blotting. Experiments involving embryo explants, in which differentiation can be experimentally controlled, will allow evaluation of the putative role of YY1-mRNPs in development of specific tissue types. Modulation of development by exogenous application or expression of key signalling molecules in the embryo, such as Wnt and BMP family members, followed by investigation of their effects on YY1-mRNPs, using the IP/Real-Time PCR approach, will determine the role of these complexes in key differentiation and patterning events. Other experiments could involve overexpression of YY1 in embryos or injection of function-blocking anti-YY1 antibodies and subsequent

quantification of levels of selected YY1-associated mRNAs and their protein products. This type of analysis of the effect of YY1 on the stability and translation of its mRNA substrates in mRNPs will finally allow direct observation of the role of YY1 in mRNPs during embryonic development. One example of the many hypotheses that have arisen from the current data, and the methods that could be used to determine its validity is as follows: YY1 has been shown to affect neural patterning in *Xenopus* (Kwon and Chung, 2003) but the precise mechanism for this effect remains elusive. Interestingly, Brain specific Casein Kinase II mRNA was identified as a substrate of YY1 in the current study. Other studies have shown CKII affects anterior-posterior neural patterning via its action on the Snail-1 protein (MacPherson et al., 2010; Thisse et al., 1993). It is possible that translational regulation by YY1 of brain specific CKII would affect anterior-posterior neural patterning via modulation of Snail-1 by brain specific CKII, a hypothesis that can now be tested experimentally.

YY1 is the first mRNP protein for which *in vivo* substrates have been identified, and the foregoing example illustrates the extreme utility of such knowledge in planning future experiments that will ultimately allow the precise role of YY1 in development to be determined. Additionally, extension of the RIP-CHIP approach to the other known mRNP proteins will facilitate development of stoichiometric models of mRNP composition and definitive patterns of maternal mRNA storage and mobilization in early development.

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